Drugs of abuse: A narrative review of recent trends in biological sample preparation and chromatographic techniques

Deborah Thais Palma Scanferla [a,*], Renata Sano Lini [a], Camila Marchioni [b], Simone Aparecida Galerani Mossini [c]

[a] *Program on Bioscience and Physiopathology, State University of Maringa, Avenida Colombo, 5790, Bloco I90 – sala 102B. Jardim Universitário, 87020-900 Maringá, PR, Brazil*
[b] *Department of Pathology, Federal University of Santa Catarina, Rua Engenheiro Agronômico Anfrei Cristian Ferreira, s/n – Trindade, Florianópolis, SC 88040-900, Brazil*
[c] *Laboratory of Toxicology, Department of Basic Health Sciences, State University of Maringa, Avenida Colombo, 5790, Bloco I90 – sala 102B. Jardim Universitário, 87020-900 Maringá, PR, Brazil*

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ABSTRACT

Introduction: Increasing numbers of drug of abuse users worldwide, combined with polyconsumption and appearance of new psychoactive drugs, have become a potential public health problem. Besides the sale of traditional herbal substances, there is evidence of the expansion of an active market for synthetic drugs and the non-medical use of prescription ones. Forensic, regulatory and health emergency organizations routinely need to use sensitive and specific analytical assays to identify and quantify drugs and the products resulting from the associations due to polyconsumption in biological samples. The appropriate choice of the sample for toxicological analyses and the preparation technique of the selected sample coupled to the chromatographic method applied are highly critical steps.
Objective: This paper aims to updating knowledge on the analysis of drugs of abuse in biological matrices.
Methodology: a narrative review of forensic analytic methodologies (sample preparation and chromatography analysis) for drugs, including options on different samples such as blood, urine, hair, bones, oral fluid and others.
Results and Discussions: An in-depth analysis is provided on variables of sample preparation techniques employed in recent years, drug concentrations in conventional and alternative specimens and characteristics of chromatographic analyses and mass spectrometry.
Conclusion: The method to be chosen by the toxicologist depends on factors such as the purpose of analysis, analyte characteristics, sample feasibility and the availability of analytical techniques. To overcome the challenges posed in drug analysis, future research efforts should be directed towards the development of multidrug methods with the application of miniaturized and automated sample preparation techniques.

Introduction

The identification of drugs of abuse in biological samples for clinical or forensic purposes constitutes a significant challenge. The increasing amount of toxic chemical substances such as classic drugs of abuse and the hundreds of new psychoactive substances (NPS), associated with complexity of biological matrices requires the continuous development of efficient and ecofriendly analytical methods [1–3].

Drugs of abuse are a major issue that threaten health and social stability. This fact challenges scientific advancements to detect its use and to establish public prevention policies. The global number of drug users is expected to grow 25 % by 2050, directly impacting road safety and public health [3–5].

It has been estimated that in the European Union about 83 million adults aged 15 to 64 years have tried illicit drugs at some phase in their lives. In addition, for every-three young Europeans who use drugs, one dies of an overdose [5,6]. In Europe, every year thousands of people suffer from acute drug-related poisoning, which requires hospital care. A total of 5,141 cases of overdose deaths involving drug poisoning have been estimated in countries of European Union in the 2019, representing an increase of 3 % compared with 2018 [6]. The World Drug Report published by the United Nations Office on Drugs and Crime, in a combined effort with the World Health Organization, reported that the number of illicit drug users has been increasing since the late 1990 s. It was estimated that 269 million people, approximately 5.4 % of the global population between 15 and 64 years old, had used illicit drugs [3].

In 2017, nearly 585,000 people died due to drug use. About 42 million "healthy" years of life have been lost (premature deaths and years spent with disability) because of drug use [3]. To make matters worse in this chaotic scenario, further challenges are presented due to changes in the global pattern of drug abuse. Coupled to the production of illicit drugs and the emergence of an extensive range of NPS, an increasing number and diverse set of chemicals which are difficult to monitor are being employed, against the polyconsumption of different classes of drugs, since they can present closely related structures [5,7]. This fact reinforces the need for constant advances in analytical methods for the identification of drugs of abuse in biological samples.

Consequently, in recent years, investments have been made to improve the availability, sensitivity and selectivity of toxicological data, and to acquire a better understanding of trends in drug use and health threats. Since, without the detection of polydrug use, these threats may be underestimated and the treatment/management of the patient may be inadequate or ineffective [8]. The trend comprises toxicological analyses and must be implemented with special awareness on the limitations imposed by the methodology and on the available biological sample. For this reason, an in-depth review is essential to assist the analyst in the careful choice of the sample and especially the technique for implementing adequate analytical protocols for drug detection in laboratories, taking into account its purpose as well as its resources and limitations.

Drugs of abuse analysis is closely related to legal aspects. The findings on illicit substances may have a causal relationship with death or damage inflicted on humans. Thus, drug analysis may be useful in determining the cause of death when violent, sudden or fatal poisoning is suspected. It may also be helpful to confirm the use of crime-facilitating drugs or to determine whether the perpetrator committed a crime under the influence of psychoactive compounds. Moreover, to check the influence on drug traffic and sports doping, analytical techniques are also necessary to analyze these substances.

Moreover, the analysis of polydrug use in the "chemsex" context is also in the spotlight in recent years. This context includes the intentional or unintentional ingestion of psychoactive and non-psychoactive drugs used mainly at raves parties and eventually followed by sexual encounters with the aim of enhancing sexual intercourse [9–12].

Highlighting the detection of polydrug use (classical and NPS), Trana

et al., developed and validated an HPLC–MS/MS method for identification and quantification of 119 analytes in blood, urine and oral fluid samples [7]. Mannocchi et al. were the only ones to investigate, to date, a wide range of drugs, also totaling 119 analytes in alternative samples, hair and nails [8].

Due to the complexity of biological matrices, the wide range of compounds that must be monitored, the size of the population under analysis, the complexities related to the time of analysis, the detection and effective quantification of drug usageare actually bioanalytical challenges for regulatory authorities and analysts worldwide. Thus, it is the toxicologists task to properly define the biological sample that will be used and the sample preparation technique and chromatographic method that will be applied. It is evident that reviews that describe and discuss recent techniques employed to determine and/or to quantify drugs may be useful to analysts since they help in the development of new methodologies in *ante* or *postmortem* analysis. Several reviews have been compiled to bring together the latest advances in drug analysis. Research carried out by Gerace et al. analyzed the different analytical approaches used for the specific investigation of synthetic opioids in *postmortem* samples from lethal cases of intoxication [13]. Abd-Elsalam et al. assessed the analytical methods developed for the investigation of cannabinoids in biological matrices from 2012 to 2018 [14]. An analysis on the chromatographic and spectroscopic methods published between 2000 and 2010 to determine club drugs, such as ketamine, gamma-hydroxybutyric acid (GHB), flunitrazepam and methamphetamine, was performed by Brown and Melton [15].

However, some studies have focused on the review of analytical methods for a wide group of drugs. Mercolini and Protti [16], for example, summarized and discussed the quantificationof new psychoactive drugs (NPS), focusing on bio-sampling. In 2018, Mogollon et al. reviewed the methods of mass spectrometry in forensic toxicology for drug identification and quantificationin biological fluids, tissues and synthetic samples, discussing new screening methodologies and other items [17].

Although Borden et al. recently discussed the advances in the last decade for the determination of illicit drugs, the authors focused on mass spectrometry and on recent advances that supported analyses that had not been met before [18].

Considering these latest reviews and gaps in the literature, current paper carried out a narrative review the available literature to answer the following question: What is the best biological sample to be used, the most adequate sample preparation and the chromatographic method to be employed in drug analysis for clinical or forensic purposes? The aim of this narrative review was, therefore (I) presents general aspects of the new methodologies in forensic drug analysis in biological matrices; (II) intends to address all stages, from the choice of the biological matrix used, conventional, miniaturized and/or automated sample preparation techniques to the characteristics of chromatographic analysis and mass spectrometry; (III) promote the acquisition and updating of knowledge, in a short period of time, on the analysis of drugs in biological matrices, bringing together different works of literature; (IV) provide a tool to easily retrieve the advantages and disadvantages of biological samples and analytical methods to assist the reader in decision making in the laboratory routine.

The results and discussion of narrative review is divided into three parts: (1) aspects related to conventional and alternative specimens will be reported, as well as about the ranges of drug range concentration found in each specimen; (2) the advantages and disadvantages of matrices preparation techniques are discussed and interpreted, underscoring variables that must be optimized to achieve adequate recovery of the analytes; (3) the characteristics of the chromatographic analyses with gas chromatography (GC) or liquid chromatography(LC) are compared.

Review of the literature

The current narrative review was performed according to the tips described by Gasparyan et al. [19]. The search for published studies was conducted in the databases PubMed (MEDLINE), Web of Science and SCOPUS. The research was conducted by four skilled operators individually. The research was time limited (from 2015 to 2020) and restricted to English. Search terms included the words "abuse drugs", "illicit drugs", "recreational drugs", "drugs", "body fluids", "fluids and secretions", "gas chromatography" and "liquid chromatography". They were combined with "AND" or "OR" to search related articles. Further publications were also included for reference verification of the articles found. Titles and abstracts were carefully read and screened for subsequent full-text review and data extraction. The search for published studies was independently conducted by four authors, while disagreements between these authors were settled through discussion.

The following inclusion criteria had to be fulfilled, with each study involving: (a) publications involving *ante* and *postmortem* cases; (b) paper containing analytical method for biological sample analysis; (c) using obligatorily gas or liquid chromatography; (d) works with forensic or clinical application; (e) articles that analyze at least one drugs. After careful selection, 79 full-text articles were included in qualitative analyses.

Datacoding tables were developed for extracting data from analytical methods (Tables 1–5). The following data were extracted: sample analyzed, analytes, concentration in biological sample, extraction technique, solvent extraction, sorbent extraction, separation system, detection system, Injection volume, stationary phase, carrier gas or liquid phase, derivatization condition and mass range.

Drugs of abuse in biological samples

Articles published for the validation of new analytical methodologies for drugs of abuse analysis generally employ the method in samples from drug users or forensic laboratories for routine analysis, or even in specific groups, as in the cases of the emerging and growing phenomenon of "chemsex" [11,12].

Most studies using the validated method involve the use of a small number of samples to show the application of the methodology under study. The exception of four studies with large populations should be underlined. Cortes et al. [20] (n = 513) collected hair and meconium samples at a hospital in Spain during one year; Grapp et al. [21] (n = 247), applied routine samples of forensic cases; Krotulski et al. [22] (n = 1233) used oral fluid samples from participants in large multi-day electronic dance music festivals in the United States; Ou et al. (2020) [23] (n = 563) collected hair samples at rehabilitation centers in China.

Analyses of articles published between 2015 and 2020 revealed that there was a period in which different alternatives specimens were analyzed, such as hair and nails for *antemortem* analyses or bones and teeth for *postmortem* ones. However, recent articles focus on the quantification of new psychoactive substances and their application to a greater number of drugs of abuse in a single method of analysis.

It has also been observed that, due to the advancement of highly sensitive techniques, the use of alternative biological samples has increased, although blood and urine are still the most explored matrices. Alternative specimens such as Dried Blood Spot (DBS), oral fluid and hair are highly important and must be carefully taken into account [7,8,12,16,24–26].

The issues concerning samples were divided into two sub-topics, namely, conventional biological samples (blood and urine) and alternative specimens (hair, oral fluid, sweat, nail, breast milk, meconium and others). For purposes of interpretation and discussion, the advantages and disadvantages of using the above biological samples for forensic analysis as well as some recommendations will be discussed in the subtopics.

Conventional biological samples

Conventional samples (blood and urine) were reported in 26 articles out of a total of 79 researches published between 2015 and 2020, representing 32.4 %. Whole blood was the preferred conventional matrix by researchers, present in 15 articles. Blood and urine were analyzed in the case of recent drug abuse as it has a short detection time span.

Table 1 shows the drugs of abuse quantified in conventional and alternative specimens forwarded by studies that presented the application of their analytical methods in real samples. It is possible to see from the data in Table 1 that there is a wide range of concentration found in different biological samples. The concentration ranges vary according to the biological matrix, but they are also correlated with the time between the use of the substance and sample collection, toxicokinetic and toxicodynamic characteristics of the substances and form of collection and conservation of the sample. Discussion about the different samples will be addressed in topic 3.2.

Alternative biological samples

Alternative specimens include biological matrices other than blood or urine. Among these, hair (n = 12, 16.92 %) and oral fluid (n = 7, 9.8 %) are the object of most included in these review studies published between 2015 and 2020 that used alternative matrizes (Table 1). Further, some *postmortem* studies present the quantification of drugs in human organs and other body fluids.

A single study reported the quantification of drugs in sweat. Gentili et al. provided results of sweat samples collected from suspects of driving under the influence of drugs [27]. The collection was performed with a commercial device and results were given in ng/collection pad (Table 1). The authors reported that the matrix was a good alternative for toxicological analysis due to the samplés easy collection and transport. On the other hand, it is not widely used routinely due to the lack of official protocols and guidelines involving the biological sample.

Since in the case of studies involving the use of drugs in pregnancy, one concern involves the exposure of the fetus to these substances, the selected articles that evaluated maternal-fetal exposure reported meconium and maternal hair as biological samples (Table 1).

Analysis of maternal hair is another way to assess fetal exposure to illicit drugs. Cortes et al. highlighted the benefit of this matrix when compared to meconium, since maternal hair is effective in retrospective detection, that is, it detects the use of illicit substances throughout all pregnancy stages [20].

Several studies have reported that drug use while breastfeeding may harm the newborn's health. Since collecting a biological sample from a child is a rather complex affair, Bertrand et al. used statistical analysis to infer the amount of Δ^9-tetrahydrocannabinol (Δ^9–THC) absorbed by babies who are breastfed by mother's milk containing the drug [28]. Estimates employed a linear regression model, stating that a 3-month-old baby, weighing 6.1 kg, would consume 3.1875 oz of milk at each feed, with 8 feedings over 24 h and oral bioavailability of Δ^9–THC equal to 6 %. The authors suggested further studies to assess the risks that drugs may present to the baby since they indicated that marijuana is a recreational drug greatly used by breastfeeding females.

In the case of *postmortem* samples, difficulties exist in interpreting the concentration of drugs in these matrices, mainly due to the lack of information in the literature on how drugs behave in different samples and on changes they undergo after death. Two articles fillthis gap in the literature and present results of concentrations obtained for U-47700 [29]. Δ^9-THC, THC-OH (11-hydroxy-Δ^9-tetrahydrocannabinol) and THC-COOH (11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol) [30], within a variety of samples obtained in *postmortem* collections, like bile, brain, heart, kigney and others (Table 1).

Klima et al. analyzed the crown, root and decayed material of teeth in three cases of dead people with a history of drugabuse and compared them with results obtained for body fluids (cardiac blood, femoral

Table 1

Aspects and reported concentration of drugs in conventional and alternative biological samples from humans, reported in the literature between 2015 and 2020 and included in this review.

Sample	Sample Aspects		Analyte	Concentration	Ref.
	Positive	Negative			
Whole blood	Recent exposure; Relationship between concentration and biological effects; Difficulty in adulterated sample; Preferred in *postmortem* cases.	Complex matrix; Collection is an invasive procedure; High viscosity; Possibility of hemolysis or clotting.	MORP R-MET S-MET	<LOQ-1.27 mg mL⁻¹ 0.16–0.64 mg mL⁻¹ 0.17–0.52 mg mL⁻¹	[98]
			AMP COC MET MORP	40.00 ng mL⁻¹ <LOQ-5500 ng mL⁻¹ 506.00 ng mL⁻¹ 10.00–5100 ng mL⁻¹	[42]
			AMP MA	37.80–38.90 ng mL⁻¹ 143.70–150.60 ng mL⁻¹	[72]
			AMP COC MET MA MDMA	30.90–116.70 ng mL⁻¹ 32.10–6003.90 ng mL⁻¹ 1494.50 ng mL⁻¹ 30.20–73.60 ng mL⁻¹ 240.30–354.90 ng mL⁻¹	[37]
			AMP MA	0.11–0.30 µg mL⁻¹ 0.95–2.26 µg mL⁻¹	[86]
			THC	0.78–8.39 ng mL⁻¹	[30]
			NPS	38.40–661.00 ng mL⁻¹	[53]
			4-CMC COC MDMA	<LOD- 2.14 ng mL⁻¹ 0.04–3.13 µg mL⁻¹ 0.04–0.09 µg mL⁻¹	[104]
			Cathinone	12.00–1200 ng mL⁻¹	[22]
			COC	<LLOQ- 4104.80 ng mL⁻¹	[38]
			MORP MET	130.00–360.00 ng mL⁻¹ 450.00–1220.00 ng mL⁻¹	[36]
			NPS MXE METH	1.32–4.04 µg L⁻¹ 7.60 mg L⁻¹ 0.08–10.60 mg L⁻¹	[101]
			COD MORP	20.91 ng mL⁻¹ 35.86 ng mL⁻¹	[127]
			AMP COC COD KET MA MORP	60.00–1100.00 ng mL⁻¹ 4.00–11.00 ng mL⁻¹ 12.00–44.00 ng mL⁻¹ 400.00 ng mL⁻¹ 212.00–24500.00 ng mL⁻¹ 12.00–559.00 ng mL⁻¹	[51]
			MORP	148 ng mL⁻¹ 100 ng mL⁻¹	[7]
Plasma	Recent exposure; Relationship between concentration and biological effects; Difficulty in adulterated sample.	Clotting factors (anticoagulant); Collection is an invasive.	4-MMC MDPV 4-MEC	86.00 ng mL⁻¹ 56.00–160.00 ng mL⁻¹ 49.00–75.00 ng mL⁻¹	[39]
			COC MET MORP	<LOQ-0.30 µg mL⁻¹ 0.10–1.30 µg mL⁻¹ <LOQ-7.90 µg mL⁻¹	[72]
Serum	Recent exposure; Relationship between concentration and biological effects; Difficulty in adulterated sample.	Collection is an invasive.	MDPV	<10.00–576.00 ng mL⁻¹	[68]
			2-MMC 3-MMC 4-MEC	12.60 ng mL⁻¹ 13.70–39.90 ng mL⁻¹ 32.10–332.00 ng mL⁻¹	[63]
			AMP COC COD MDMA MDPV MA METH MORP	5.00–549.00 µg/L 5.00–2444.00 µg/L 6.70–19.00 µg/L 13.30–341.00 µg/L 5.00–576.00 µg/L 123.00–254.00 µg/L 14.00 µg/L <5.00–972.00 µg/L	[21]

Table 1 (*continued*)

Sample	Sample Aspects		Analyte	Concentration	Ref.
	Positive	Negative			
			α-PVP	10.00 μg/L	
			NPS	0.10–3.70 μg/L	
DBS	Recent exposure; Relationship between concentration and biological effects; Difficulty in adulterated sample; Non-invasive collection method; No need for anticoagulant or plasma separation; Easy shipment of sample Small sample volume; Stability at room temperature.	Humidity variation; Low sensibility for some analytes; Hematocrit percentage can bias the quantification analysis; Difficulty in standardizing the sample volume.	AMP	30.9 – 116.7 ng mL^{-1}	[25]
			MA	30.2 – 73.5 ng mL^{-1}	
			MDA	26.9 – 48.4 ng mL^{-1}	
			MDMA	240.3 – 354.9 ng mL^{-1}	
			COC	32.1 – 6003.9 ng mL^{-1}	
			BZE	44.9 – 748.8 ng mL^{-1}	
			THC	Traces ng mL^{-1}	
			THC-OH	Traces – 15.0 ng mL^{-1}	
			THC-COOH	Traces – 23.8 ng mL^{-1}	
			EDDP	25.1 ng mL^{-1}	
			MET	1494.5 ng mL^{-1}	
Urine	Metabolites that are frequently in greater quantity. Non-invasive collection method. Availability in large quantities. Detection time span longer than blood. Less endogenous interferences. Most employed matrix in cases of non-targeted screening.	Easy sample adulteration. Low correlation with the effect. Degree of hydration of the individual which may interfere in analyte concentration.	4-MEC	46.00–126.00 ng mL^{-1}	[39]
			MDPV	114.00–148.00 ng mL^{-1}	
			4-MMC	113.00 ng mL^{-1}	
			THC	1.25–32.20 ng mL^{-1}	[30]
			NPS	0.24 μg mL^{-1}	[29]
			AMP	131.00–276.00 ng mL^{-1}	[154]
			MDMA	110.00 ng mL^{-1}	[128]
			COC	< LOQ- 25.90 ng mL^{-1}	[47]
			MORP	58 – 380 ng mL^{-1}	[7]
			COD	14 – 150 ng mL^{-1}	
			6-MAM	15 – 120 ng mL^{-1}	
Hair	Wide detection time span; Non-invasive collection; Easy transport and storage; Stableness; permits a retrospective analysis with an accurate history of drug use.	External decontamination process (spraying or grinding in a homogenizer).	COC	153.00 μg mg^{-1}	[59]
			COD	13.00–13.10 μg mg^{-1}	
			MORP	2.30–3.10 μg mg^{-1}	
			CBD	0.46–1.83 ng mg^{-1}	[20]
			COC	0.10–3.91 ng mg^{-1}	
			THC	0.17–1.15 ng mg^{-1}	
			CBN	0.05–0.33 ng mg^{-1}	
			MDMA	0.33 ng mg^{-1}	
			NPS	0.14 ng mg^{-1}	[29]
			DMMC	572.60–2800.00 pg mg^{-1}	[99]
			4-FMC		
			4-MEC	41.10–45.60 pg mg^{-1}	
			α-PHP	591.00–2200.00 pg mg^{-1}	
			α-PVP		
			synthetic cathinone	3600.00–4700.00 pg mg^{-1}	
				24.40–52.80 pg mg^{-1}	
				11.00 pg mg^{-1} –6200.00 pg mg^{-1}	
			AMP	74.20–369.20 pg mg^{-1}	[48]
			COC	30.10->2000.00 pg mg^{-1}	
			COD	1283.10 pg mg^{-1}	
			KET	14.20->2000.00 pg mg^{-1}	
			LSD	11.10 pg mg^{-1}	
			MDMA	>2000.00 pg mg^{-1}	
			MA	328.80->2000.00 pg mg^{-1}	
			MORP	847.20 pg mg^{-1}	
			COC	510.00 pg mg^{-1}	[49]
			MDMA	80.00 pg mg^{-1}	
			MET	680.00 pg mg^{-1}	
			MORP	250.00 pg mg^{-1}	
			THC	220.00 pg mg^{-1}	
			NPS	0.06–15300.00 pg mg^{-1}	[61]
			GHB	0.34–4.17 ng mg^{-1}	[88]
			THC-COOH	0.10–27,30 pg mg^{-1}	[129]
			AMP	0.004–7.15 ng mg^{-1}	[23]
			MA	0.026–57.51 ng mg^{-1}	
			AMP		[8]
			MDMA	100 pg mg^{-1}	
			KET	77.2 – 5500 pg mg^{-1}	
			BZE	27.3–900 pg mg^{-1}	
			COC	12–9000 pg mg^{-1}	
			MORP	900 – 2100 pg mg^{-1}	
			COD	200 – 600 pg mg^{-1}	

Table 1 (*continued*)

Sample	Sample Aspects		Analyte	Concentration	Ref.
	Positive	Negative			
			6-MAM	900–2100 pg mg^{-1}	
			MET	1300 – 1400 pg mg^{-1}	
			THC	23.4 – 9800 pg mg^{-1}	
			NPS	400–2300 pg mg^{-1}	
				50–3200 pg mg^{-1}	
			COC	12.36–86.76 ng mg^{-1}	[12]
			BZE	1.64 – 7.03 ng mg^{-1}	
			KET	1.59 – 5.03 ng mg^{-1}	
			GBL	3.2 – 12.1 ng mg^{-1}	
			MDA	0.09 – 0.1 ng mg^{-1}	
			4- MEC	-	
			Methylone	-	
			Methcatinone	-	
			AMP	1.38 ng mg^{-1}	
			MA	1.47 – 10.9 ng mg^{-1}	
Oral Fluid	Easy collection mode; Non-invasive collection method; Difficulty to adulterate; Good correlation with urine; Shorter detection span;Expectoration (collect) .	High viscosity; complex composition;Commercial devices (higher costs for collection and not possible to aliquot the collected sample) .	4-MMC	18.00–40.00 ng mL^{-1}	[39]
			COD	6.0–122.6 ng mL^{-1}	[43]
			Hydrocodone cathione	1.9–319.9 ng mL^{-1} 12.60–1377.00 ng mL^{-1}	[22]
			4-FA	281.60–378.20 ng mL^{-1}	[22]
			α-PVP		
			MDMA	87.80–1301.00 ng mL^{-1}	
			METH		
			NPS	4.00->10000.00 ng mL^{-1}	
				40.30–7795.00 ng mL^{-1}	
				4.40–4105.00 ng mL^{-1}	
			CBD	4.50–15.30 ng mL^{-1}	[44]
			CBN	<LOQ-66.20 ng mL^{-1}	
			THC	10.00–655.20 ng mL^{-1}	
			AMP	52.80 ng mL^{-1}	[67]
			COC	18.20–35.10 ng mL^{-1}	
			MDMA	46.20–64.70 ng mL^{-1}	
			MORP	68.80–79.10 ng mL^{-1}	
			THC	12.40–22.40 ng mL^{-1}	
			BZE	0.3 – 17 ng mL^{-1}	[7]
			COC	0.2 – 110 ng mL^{-1}	
			MORP	0.1 – 1 ng mL^{-1}	
			6-MAM	0.2–1 ng mL^{-1}	
			Synthetic cannabinoids	0.29 – 8.10 ng mL^{-1}	[26]
Sweat	Non-invasive collection; Repeated sampling;Severely decreases the risk of contamination (people suf-fering of infectious diseases) .	Sample recovery; Not allow the measure-ment of collected sweat volume.	COC	10.10–600.90 ng pad^{-1}	[27]
			MDMA	10.00 ng pad^{-1}	
			THC	0.50–14.10 ng pad^{-1}	
Nails	Wide detection time span; Non-invasive collection; easy transport and storage; stableness; May be an alternative or a complementary analysis of the hair.	The literature fails to report many studies; Lack of knowledge on the impregnation mechanism of the drug in the matrix.	MET	7.50–78.00 ng mg^{-1}	[71]
			MDA	10 – 359 pg mg^{-1}	[8]
			MDMA	29 – 7000 pg mg^{-1}	
			KET	65 – 1307 pg mg^{-1}	
			BZE	14.278 – 4968 pg mg^{-1}	
			COC	17 – 69 pg mg^{-1}	
			THC	10 – 710 pg mg^{-1}	
			MA	17–71 pg mg^{-1}	
			NPS		
			COC	0,34–690,4 ng mg^{-1}	[12]
			BZE	0,12–174,0 ng mg^{-1}	
			KET	0,08–20,62 ng mg^{-1}	
			GBL	0,03–50,4 ng mg^{-1}	
			MDA	0,2–0,79 ng mg^{-1}	
			4- MEC	4,85–1.670,0 pg mg^{-1}	
			Methylone	2,7–50,8 pg mg^{-1}	
			AMP	0,08–0,95 ng mg^{-1}	
			MA	0,23–1,14 ng mg^{-1}	
			NPS	0,1–52,3 ng mg^{-1}	
			butylone	72,0 pg mg^{-1}	
			GHB*	0.3 – 3.8 ng mg^{-1}	[11]
			GHB glucuronide* *endogenous	0.08 – 0.243 ng mg^{-1}	

Table 1 (*continued*)

Sample	Sample Aspects		Analyte	Concentration	Ref.
	Positive	Negative			
Breast milk	Exposure of infants to substances during breastfeeding; Simple and non-invasive collection.	High lipid and protein content; Change in composition during the postpartum period.	CBD	1.32–8.56 ng mL^{-1}	[28]
			THC	1.01–323 ng mL^{-1}	
Meconium	Determining prenatal exposure to several drugs; Easy collection;Wide detection time span (12 weeks of gestation).	Complex matrix that requires treatment before analysis.	COC	3.72–3.75 ng g^{-1}	[20]
			AMP	LOQ-2220.00 ng g^{-1}	[83]
			MA	18.00–13325.00 ng g^{-1}	
Bones	Attractive matrix for *postmortem* case analysis.	Complex matrix; Influence the interpretation of toxicological analysis.	MET	14.00–28.00 ng g^{-1}	[36]
			MORP	3.00–7.00 ng g^{-1}	
			COC	0.48–3.74 ng g^{-1}	[33]
			COD	1.01–34.62 ng g^{-1}	
			MORP	6.80–172.70 ng g^{-1}	
Carious material	Attractive matrix for postmortem case analysis; Drug accumulation in long-term exposure; Retrospective detection span; Hardly contaminated by external factors;	Complex matrix.	AMP	8.70 pg mg^{-1}	[31]
			COD	7.10–120.00 pg mg^{-1}	
			MET	1.00–520.00 pg mg^{-1}	
			MORP	44.00–440.00 pg mg^{-1}	
			NPS	0.13 pg mg^{-1}	
Tooth crown	Window of detection seems to overlap those for body fluids and hair.		AMP	12.00 pg mg^{-1}	
			COD	10.00 pg mg^{-1}	
			MET	0.54–120.00 pg mg^{-1}	
			MORP	5.80–30.00 pg mg^{-1}	
Tooth root			AMP	4.70 pg mg^{-1}	
			COD	1.20–2.50 pg mg^{-1}	
			MET	1.10–7.00 pg mg^{-1}	
			MORP	5.50–8.00 pg mg^{-1}	
Non-mineralized dental biofilm			AMP	33.00–1400.00 pg mg^{-1}	[32]
			COC	18.00 pg mg^{-1}	
			COD	44.00–290.00 pg mg^{-1}	
			MDMA	19.00 pg mg^{-1}	
			MORP	43.00–490.00 pg mg^{-1}	
Bile	Attractive matrix for *postmortem* case analysis; Suitable supplemental specimens.	Complex matrix.		0.78–50.40 ng g^{-1}	[30]
Brain				1.34–43.60 ng g^{-1}	
Heart				1.70–472.00 ng g^{-1}	
Kidney			THC	0.99–450.00 ng g^{-1}	
Liver				22.30–52.20 ng g^{-1}	
Lung				1.82–151.00 ng g^{-1}	
Muscle				1.19–377.00 ng g^{-1}	
Spleen				0.78–20.00 ng g^{-1}	
Bile fluid	Attractive matrix for *postmortem* case analysis; Suitable supplemental specimens.	Complex matrix.	NPS	2.30 µg mL^{-1}	[29]
Cerebrospinal fluid				0.40 µg mL^{-1}	
Femoral blood				0.29 µg mL^{-1}	
Gastric contentes				0.57 µg mL^{-1}	
Heart blood				1.25 µg mL^{-1}	
Liver				9.90 µg mg^{-1}	

where: (1-(4-chlorophenyl)-2-(methylamino)-1-propanone) (4-CMC); 6-Monoacetylmorphine (6-MAM); alpha-pyrrolidi- nohexiophenone (α-PHP); alpha-pyrrolidinopentiophenone (α-PVP); amphetamine (AMP); benzoylecgonine (BZE); cannabidiol (CBD); cannabinol (CBN); cocaine (COC); codeine (COD); fluoromethcathinone (4-FMC); gamma-hydroxybutyrate (GHB); ketamine (KET); Lysergic acid diethylamide (LSD); mephedrone (4-MMC); methadone (MET); methamphetamine (MA); *methoxetamine* (MXE); 3,4-methylenedioxymethamphetamine (MDMA); 3,4-dimethyl- methcathinone (DMMC); 3,4-methylenedioxy-pyrovalerone (MDPV);2-(methylamino)-1-(2-methylphenyl)-1-propanone, monohydrochloride (2-MMC);3-Methylmethcathinone (3-MMC); 4-Fluoroamphetamine (4-FA); methylethcathione (4-MEC); methylone (METH); morphine (MORP); Δ9-Tetrahydrocannabinol (THC); (R)-enantiomer methadone (R-MET), (S)-enantiomer methadone (S-MET), New psychoactive substances (NPS) = *N*-ethyl pentylone, Fentanyl, 25B-NBOMe, 25C-NBOMe, 25I-NBOMe, 5-MeO-MiPT, AB-CHMINACA, MDMB-CHMICA, U-47700, 5F-AKB-48,AB-FUBINACA, AKB-48, JWH-210, PB-22, XLR-11, 4-FA, Hydrocodone, Butylone, Dibutylone, Dimethylone, Ethylone.

venous blood, serum, urine, stomach contents) and hair [31]. The authors concluded that preliminary results suggested that the tooth detection span lies between body fluids and hair spans. They also observed that teeth enamel may be considered as a protective barrier for tooth contamination by the drug in oral fluid.

Henkel et al. analyzed dental biofilms from three *postmortem* cases and observed that this matrix may be an alternative to conventional samples, such as blood, since results demonstrated that dental biofilm has a long-lasting detection span when compared to that from blood and oral fluid [32].

In a paper on toxicological analyses on fresh and one-year-old buried human bones, Orfanidis et al. demonstrated the difficulty in setting a deadline for the detection of drugs in bones [33]. However, the matrix offers a means of detecting drugs when no other material is available for analysis, especially when there is a long time elapsed between death and sampling, or even in cases of significant putrefaction, skeletal tissues may be the only source of information [34]. The authors reported that it was not possible to estimate the time of drug exposure but emphasized that its presence in the bone is indicative of previous exposure to the substance [33].

Post-mortem toxicological investigation in the biological matrix of human bone may result in different drug levels, which may be influenced by some parameters (route of administration, dose, consumption patterns, time between last exposure and death) unknown in forensics cases [34,35]. In addition, different bones can result in different drug levels being found, although there are no human studies looking at which bone is best suited for *post-mortem* toxicology analyses [35].

In 2019, Fernández-Lopez and colleagues developed and validated

Table 2

Sample preparation by Liquid-Liquid Extraction (LLE) and miniaturized descendants reported in the selected literature between 2015 and 2020 to extract drugs from biological sample.

Analyte	Sample	Extraction technique	Solvent Extraction	Ref.
Synthetic cannabinoids	Oral Fluid (400 µL)	LLE	6 mL (2X 3.0 mL of hexane:ethylacetate mixture (9:1, v/v).	[26]
COC, CE, BEG, EME, AMP, MA, MDA, MDMA, MET, LSD, KET, EDDP, MORP, 6-MAM, NK buprenorphine, and fentanyl and analogues), benzodiazepines, Z-compounds	blood samples (50 µL)	DLLME	100 µL of chloroform and 250 µL of methanol	[42]
AMP, MA, MDA, MDMA, KET, MET, 4-MMC and methcathinone	urine (12 mL) and blood (1 mL)	HF-LPME and UA-LDS-DLLME	~10 µL of toluene (HF-LPME) 100 µL of toluene (UA-LDS-DLLME)	[87]
MORP, COD, 6-MAM, MET, EDDP, COC, BEG, THC, THC-OH, THC-COOH, AMP, MA, MDMA, MDA and MDPA	DBS (30 µL)	LLE	990 µL methanol	[37]
AMP, MA, MDMA, MDEA, MDA, KET, NK, MORP, COD, COC, BEG, LSD, MET, 6-MAM, THCA, PMA, PMMA, PCP, 4-MMC, METH, 2C-B and NPS	urine (1 mL)	LLE	0.5 mL 1.5 Msodium buffer (pH 9.5) and 3 mL ethylacetate	[135]
AMP, MA, KET, NK, LSD, PMA, PMMA, MDA, MDMA, MDEA, MET, COC-OH, COD, MORP, 2C-B, PCP and benzodiazepines, barbiturates.	DBS (25 µL)	LLE	200 µL of 80 %acetonitrile	[69]
MDPV	serum (1 mL) and urine (3 mL)	LLE	10 mL (2x 5 mL diethyl ether/ethyl acetate (1:1 vol/vol) for urine and serum (LLE alcaline) 10 mL of a mixture of ethyl acetate, dichloromethane and 2-propanol (3:1:1 v/v/v) for urine (LLE acid)	[68]
AMP, MA, MDA, MDMA, MET, KET and 4-MMC	whole blood (200 µL)	UA-DLLME	200 µL dichloromethane (extraction) and 1.3 mL methanol (disperser)	[86]
25B-NBOMe and 4-CMC	postmortemblood (200 µL)	LLE	2 mL of ethyl acetate	[52]
AMP, MA, MDA, MDMA, MDPV, COC, BEG, EME, MORP, COD, EDDP, MET, α-PVP and others NPS	serum (1 mL- GC–MS) (0.2 mL- LC-QTOF-MS)	LLE	2 mL (2x 1 mL diethyl ether/ethyl acetate (1:1 vol/vol) for LC-QTOF-MS *10 mL (2x 5 mL diethyl ether/ethyl acetate (1:1 vol/vol) p/ GC–MS	[21]
AMP, MA, MDMA, MDEA, MDA, COC, BEG, MORP, COD, 6-MAM	non-mineralized dental biofilm (plaque) (2 mg)	LLE	500 µL of acetonitrile	[32]
MORP, 6-MAM, MET, EDDP and EMDP	nails (30 mg)	LLE	3 mL (3x 1 mL of methyl *tert*-butyl ether (MTBE) (99.5 %).	[71]
AMP, MA, MDA, MDMA, MDEA, MORP, 6-MAM, COD, MET, COC, EME, BEG, THC-COOH, antidepressants, benzodiazepines, antipychotics, and anticholinergic.	bones (1 g)	LLE	3 mL of methanol and 12.5 µL of ammonium hydroxide (13.4 M)	[33]
AMP, MA, BEG, EDDP, MDA, MDMA, MET, COD, MORP, COC	DBS (50 µL)	LLE	3 mL methanol/acetonitrile (3:1, v/v)	[70]
AMP, MA, 6-MAM, MDA, MDMA, COC, NCOC, AEME, CE, COD, MORP, THC-COOH, benzodiazepines	blood samples (100 µL)	LLE	800 µL of an acetonitrile/methanol mixture (80:20, *v/v*)	[58]
AMP, MA, MDMA, MDPV, α-PVP, 4-MMC, METH, butylone, flephedrone, and naphyrone	meconium (0.2 g)	SALLE	1 mL (2x de 500 µL) of acetonitrile. *50 mg of ammonium formate and ammonium bicarbonate (2:3, w/w)	[83]
MDMA, AMP, COC-OH, CE and BEG	DOFS (50 µL)	LLE	200 µL of acetonitrile, ammonium acetate buffer (14 mM), and methanol (55:35:10 v/v).	[75]
EME, MORP, COD, AMP, MA, MDA, 6-MAM, MDMA, KET, BEG, MDEA, MDPV, NCOC, α-PVP, THC, EDDP, THC-COOH, THC-OH, CBD, CBN, MET, 2-FMC, MESC, METH, MABP, 2C-H, 2-MeOMC, MBDB, 4-MMC, d-EtC, 4-MEC, MXE, 2C-B, *N*-BUP, 2C-T-4, PCP, BUP, and others syntethic canabinoids.	hair (10 mg)	PLE-dLLME	mixture of formate buffer 0.15 M (pH 3.5) /2-Propanol (80:20, v/v) for PLE. 200 µL of chloroform (extraction solvente) and 500 µL 2-propanol (dispersing solvente) for DLLME.	[49]
COC, BEG, CE, COC-OH	diluted urine (1.5 mL)	HFRLM	20 µL of a mixture of hexane: dichloromethane: ethyl acetate (1:1:1, v/v/v)	[47]
GHB	hair (50 mg)	UA-LDS-DLLME	1 mL saturated ammonium dihydrogen phosphate solution and 180 µL ethyl acetate.	[88]

where: 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH); 4-methoxyamphetamine (PMA); (1-(4-chlorophenyl)-2-(methylamino)-1-propanone) (4-CMC); 11-nor-9-carboxy-Δ⁹-tetrahydrocannabinol (THC-COOH); 2,5-Dimethoxy-4-isopropylthiophenethylamine (2C-T-4); 2,5-Dimethoxyphenethylamine (2C-H); 2-ethyl-5-methyl-3,3 diphenylpyrrolidine (EMDP); 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP); 2-Fluoromethcathinone (2-FMC); 2-Methoxymethcathinone (2-MeOMC); 2- (4-iodo-2,5-dimethoxyphenyl) -N - [(2 methoxyphenyl) methyl] ethanamine (25B-NBOMe); 3,4 methylenedioxyamphetamine (MDA); 3,4 methyl-enedioxymethamphetamine (MDMA); 3,4-methylenedioxy- *N*-ethylamphetamine (MDEA); 3,4-methylenedioxy-*N*-propyl-amphetamine (MDPA); 3,4-methylenediox-ypyrovalerone (MDPV); 4-Bromo-2,5-dimethoxyphenethylamine (2C-B); 4-Methylethcathinone (4-MEC); 6-monoacetylmorphine (6-MAM); alpha-pyrrolidinopentiophenone (α-PVP); amphetamine (AMP); Anhydroecgonine methyl ester (AEME); benzoylecgonine (BEG); buphedrone (MABP); buprenorphine (BUP); cannabidiol (CBD); cannabinol (CBN); cocaethylene (CE); cocaine (COC); codeine (COD); diethylcathinone (d-EtC); Dispersive Liquid Liquid Microextraction

(DLLME); Dried Blood Spot (DBS); Dried Oral Fluid Spots (DOFS); ecgonine methyl ester (EME); Gama-hidroxibutirato (GHB); Hollow Fiber Liquid-Phase Microextraction (HF-LPME); Hollow-Fiber Renewal Liquid Membrane extraction (HFRLM); hydroxy–cocaine (COC-OH); ketamine (KET); Liquid-Liquid Extraction (LLE); Lysergic acid diethylamide (LSD); mephedrone (4-MMC); mescaline (MESC); methadone (MET); methamphetamine (MA); methoxetamine (MXE); methylone (METH); morphine (MORP); *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine (MBDB); norbuprenorphine (*N*-BUP); norcocaine (NCOC); norketamine (NK); *para*-methoxymethamphetamine (PMMA); phencyclidine (PCP); Pressurized Liquid Extraction (PLE); Salting-out Assisted Liquid-Liquid Extraction (SALLE); tetrahydrocannabinol carboxylic acid (THCA); Ultrasound-Assisted Dispersive Liquid–Liquid Microextraction (UA- DLLME); Ultrasound-Assisted Low-Density Solvent Dispersive Liquid-–Liquid Microextraction (UA-LDS-DLLME); Δ9-Tetrahydrocannabinol (THC).

methodologies for the identification and quantification of medications for hypertension and antidepressants, respectively [34,35]. The bones chosen for the study were rib bones and the results indicated the utility of human ribs as a toxicological matrix. The methodology was applied to two and eleven authentic samples of whole blood and bones, respectively. All positive blood cases also showed positive results in the bones, despite the analyte concentrations found to be lower in the bones, demonstrating the effectiveness of the validated methods [34,35].

Fernandez-Lopez et al. showed that, despite medical progress, there were no protocols for drug analysis in the bone matrix and concluded that their results were preliminary and needed further research to study the different factors that influenced drug quantification (opioids and cocaine) in this matrix [36].

Recent articles address DBS as an alternative for live cases. This collection method does not require a trained professional, it is not a very invasive procedure and reduces the risk of accidents with biological material since it involves a small punch in the finger that may be done with a single-use lancet and is easy to transport, store and preserve due to drying [37–39]. On the other hand, the sample collects a very small volume of the material and, therefore, sensitive analysis methods are required [38]. Especially when it comes to analytes such as cannabinoids, due to the fact that they are the most challenging analytes in DBS, because, once consumed, they quickly disappear from the blood [25].

In a validated methodology for the simultaneous determination of a panel of psychoactive drugs in DBS, totaling 23 analytes and biotransformation products, two authentic positive urine samples (immunoassay) from the same individual at the same collection time, DBS analysis resulted in levels traces of THC. However, in one of these cases, although THC was below the LOQ, both THC-OH and THC-COOH were quantified [25].

Another device is the Volumetric Absorptive Microsampling (VAMS^{TM}), marketed for collecting an exact volume ($\pm$10 μL) and designed for biological whole blood matrix [40]. Mercolini et al. (2016) [39] showed promising results in the use of this device for other matrices, such as plasma, urine and oral fluid, for analysis of methylone, ethy-lone, butylone, mephedrone, 4-methylethcathinone and 3,4-methylenedioxypyrovalerone, by liquid chromatography tandem mass spectrometry (LC-MS/MS).

VAMS sampler, a highly hydrophilic absorbent material (Fig. 1) [41], underwent several experiments to develop effective and standardized protocols for the matrices mentioned. Parameters developed comprised exposure time, drying time, temperature, humidity and exposure to light. After sampling in the volumetric absorptive microsampling device, the analytes were desorbed from the tip (dry matrices) and several tests were carried out to identify a suitable solvent for this purpose. Pure methanol was indicated due to its greater extraction efficiency when compared to acetonitrile mixed with water and ethyl acetate [39].

Most authors do not mention *cut-off* rates for the analytes analyzed in a real sample, where rates obtained for the limit of quantification (LOQ) and limit of detection (LOD) under study for analytes in whole blood were below the existing *cut-offs* [42]. In oral fluid, authors cite the *cut-off* rates of 15 ng mL^{-1} for opioids [43] and 2 ng mL^{-1} for Δ^9 –THC [44]. Bassoti et al. (2020) applied the recommended maximum *cut-off* values for confirmation tests in oral fluid from European Guidelines. The values veries from 1 to 20 ng mL^{-1} for different analytes [45,46]. In the case of urine, a reference of 150 ng mL^{-1} for cocaine was reported [47].

Hair was the biological sample with the highest number of *cut-offs* reported by authors. Shin et al. followed the Society of Hair Testing

(SoHT) guide, with rates of 200 pg mg^{-1} for amphetamine, methamphetamine, codeine and morphine, and 500 pg mg^{-1} for cocaine [48]. Vincenti et al. also followed the SoHT as a guide, with rates 0.2 ng mg^{-1} for opioids and 0.5 ng mg^{-1} for cocaine and Δ^9 –THC [49]. Besides SoHT, Ou et al. followed the Workplace Drug Testing Society (EWDTS) and Substance Abuse and Mental Health Services Administration (SAMHSA), with rates 0.3 ng mg^{-1} for amphetamine and methamphetamine [23].

There is a reported difficulty in proving consumption of GHB, due to the dual nature of the endogenous neurotransmitter and the exogenous pharmacologically active compound [11,50]. For the first time, Busardó et al., after validating the UHPLC-MS/MS methodology applied to 90 authentic samples from a general population not consuming GHB, proposed preliminary cut-off values of 5.0 ng mg^{-1} nail for endogenous GHB and 0.5 ng mg^{-1} for endogenous GHB-Gluc in the general population [50].

Hair has a higher concentration range, with the lowest rates for analytes, when compared to the other matrices. This type of sample has higher ranges, probably because it has a greater detection span (months or years, depending on the hair size). On the other hand, the concentrations in plasma and serum found for the analytes are higher. The concentration of drug ranges in whole blood and DBS are similar, since dried blood spot is a whole blood sample with a different collection method [51].

A complementary sample to the hair and also an alternative for the retrospective determination of the consumption of psychotropic drugs in forensic contexts, mainly post-mortem, are the nails [8,11,12]. A study by Mannocchi et al. reports that nails can provide additional information. In this study, four different cathinones were not found in hair, however, they were detected in the individual's nails [8]. This result corroborates another study carried out by Busardò et al., who concluded that some classes of drugs were better identified in nails than in hair in two cases of authentic samples [12].

Figure 2-A represents the frequency of the samples used in the studies selected for this review. The whole blood is the main sample used, as well as its derivatives, serum and plasma. The choice of the whole blood sample is justified due to the greater interest in recent detection of drugs of abuse and the relationship between concentration and effect. Then there is a higher frequency of alternative samples (others), especially in the postmortem application (Sweat, nails, breast milk, bones, carious material, tooth crown, tooth root, non-mineralized dental, biofilm, bile, brain, heart, kidney, liver, lung, muscle, spleen, bile fluid, cerebrospinal fluid, femoral blood, gastric contentes, heart blood, liver), which are mentioned, each one, by only one study. Sequentially, there is a higher frequency of samples of urine, hair, oral fluid and dry samples (dried blood spot and dried urine spot).

Sample preparation

Due to rapid changes and a highly competitive technological environment, there are currently continuous challenges in the bioanalysis field. The preparation of a biological sample prior to analysis by instruments is highly important for the identification and quantification of drugs in biological samples.

Choice will mainly depend on the biological matrix and on the physicochemical properties of the psychoactive substance(s) to be analyzed [52,53]. Fig. 3 compares the main techniques used in recent years.

Table 3

Sample preparation by Solid-Phase Extraction (SPE) and miniaturized descendants reported in the literature between 2015 and 2020 included in this review to extract drugs from biological sample.

Analyte	Sample	Extraction technique	Solvent extraction	Sorbent extraction	Ref.
GHB GHB glucuronide	nail (25 mg)	SPE	0.5 mL multimatrix eluente (Comedical®)	Oasis PRiME HLB (reversed-phase)	[15]
Opiates, cocainics, THC, amphetamine type substances, ketamine, norketamine, GHB, GLB, methylone, butylone, mephedrone, 4 methylethcathinone and NPS	nail (25 mg) and hair (25 mg)	SPE	0.5 mL multimatrix eluente (Comedical®)	Oasis PRiME HLB (reversed-phase)	[12]
THC-COOH, CBD, CBN, Steroids, Narcotics, Stimulants and other drugs	oral fluid (*in situ* – 5 min)	TF-SPME	1 mL acetonitrile: water (80:20, v/v)	hydrophilic lipophilic balanced particles (HLB) and C18 particles	[109]
MORP,6-MAM and MET	whole blood samples (0.6 g)	SPE	5 mL aqueous ammonia water in methanol (5 % volume fraction; 2x1mL)	C18 column	[97]
KET and NK	urine (0.25 mL) and plasma (0.25 mL)	MEPS	0.1 mL of 6 % ammonia in methanol (urine) 0.1 mL of 3 % ammonia in methanol (plasma).	mixed mode (M1) cartridges (4 mg; 80 % C8 and 20 % SCX)	[74]
AMP, MA, MDA, MDMA, MDEA, MBDB, THC, MET, COC and CE	sweat	HS-SPME	200 µL (1 M HCl)	polydimethylsiloxane (100 µm)	[27]
MORP, 6-MAM, MET, COC, CE, BEG, MDPV, EDDP, 4-MMC and METH	plasma (300 µL)	digitally programmed microextraction by packed sorbent (eVol®-MEPS)	200 µL dichloromethane: 2-propa-nol: ammonium hydroxide (78:20:2, v/v/v).Washing using 150 µL water: methanol (90:10, v/v)	C8/SCX system	[72]
MDPV	serum (1 mL)	SPE	2 mL of dichloromethane: 2-propanol: ammonia solution (25 %) (40:10:1, v/v/v)	CHROMABOND Drug Columns (Silica with bifunctional modification - C8, 200 mg, 3 mL)	[68]
THC, 11-OH-THC and THC-COOH	postmortem fluid (0.5 mL) and tissue specimens (1.5 g)	SPE	2 mL of hexanes (for THC). 2 mL 50:50 hexanes: ethyl acetate (for 11-OH-THC and THCCOOH).	SPE Clean Screen THCColumns (NAX + C18, 200 mg, 10 mL)	[30]
COC and MDMA	blood (1 mL)	d-SPE	2 mL acetonitrile	Supelclean PSA (primary and secondary amine, 50 mg) and magnesium sulfate anhydrous (150 mg)	[104]
MORP, 6-MAM and COD	neat oral fluid (750 µL)	SPE	2 mL of dichloromethane: methanol:ammonium hydroxide (78:20:2 (v:v:v))	SPEware Cerex ClinII (polymeric, 35 mg)	[43]
MORP, 6-MAM, MET, METH, EDDP and EMDP	nails (30 mg)	SPE	2 mL (2x 1 mL of a 5 % Ammonium hydroxide /methanol solution)	MCX columns (10 mg, 1 mL)	[68]
4-FMC, 4-MEC, α-PVP, α-PHP, MDPV, DMMC, others NPS	hair (20 mg)	SPE	2 mL dichloromethane-isopropanol mixture (8:2 v/v) with 2 % ammonia solution	Bond Elut Certify I (mixed mode, 200 mg)	[99]
AMP, MA, MDA, MDMA, MBDB and MDEA	urine (200 µL)	MEPS	solution containing 2 % ammonium hydroxide in acetonitrile (4 cycles of 100 µL).	C18 cartridge	[128]
MORP, 6-MAM and COD	blood (250 µL)	MEPS	2.36 % ammonium hydroxide in methanol (11 cycles of 250 µL).	mixed mode (M1) cartridges (80 % C8 and 20 % SCX)	[127]
THC, CBD, CBN and others syntethics cannabinoids	oral fluid (1 mL)	DI-SPME *inline	–	polydimethylsiloxane (100 µm)	[44]
MORP, 6-MAM, MET and BEG	human bone (300 mg)	SPE	2 mL of Dichloromethane: Isopropanol:ammonia (78:20:2, v/	CleanScreen PKG50 (3 cc, 200 mg)	[36]

Table 3 (*continued*)

Analyte	Sample	Extraction technique	Solvent extraction	Sorbent extraction	Ref.
			v) .		
COC, MA, α-PVP, α-PHP, MPHP, METH, MDPPP, MDPBP, 2C-E, mexedrone, butylone and others NPS	oral fluid (200 μL)	DPX	500 μL (5x 100 μL 1 % acetic acid in metanol)	Monolithic polymer based on poly (methacrylic acid-*co*-ethylene glycol dimethacrylate) (MAA-*co*-EDMA)	[101]
6-APB, METH, and others NPS	Blood (1 mL) and urine (1 mL)	SPE	3 mL dichloromethane: isopropanol:ammonia (78:20:2, v/v/v)	Clean Screen ® ZSDAU020 (BCX + C8)	[98]
MORP, 6-MAM, MET, BEG, COC, CE, AMP, EDDP, MDA, MDEA, MDMA, MA, KET, LSD and COD	hair (10 mg)	dSPE	1 mL methanol	Sorbent = Q-sep® dSPE tubes containing MgSO4 and C18-EC	[48]
6-MAM, MORP, COD, AMP, MA, MDA, MDMA, COC, BEG, CE, EME, KET, NK, THC-COOH and and others	postmortem whole blood (1 mL)	SPE	hexane: ethyl acetate (1:1, v/v) and 3.0 mL of dichloromethane:isopropanol: ammonium hydroxide (78:20:2, v/v)	SPE Clean Screen® (BCX + C8)	[51]
Synthetic opioids	hair (20 mg)	SPE	1 mL of methanol and ammonia (95:5, v/v)	MCX cartridges (60 mg, 3 cm^3)	[100]
THC-COOH	hair (20 mg)	Column switching	2 mM ammonium formate/0.2 % formic acid and 2 mM ammonium formate/0.2 % formic acid in acetonitrile (59:41, v/v)	C18 column	[129]

where: 4′-methyl-α-pyrrolidinohexiophenone (MPHP); 11-hydroxytetrahydrocannabinol (11-OH-THC); 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH); 2-ethyl-5-methyl-3,3 diphenylpyrrolidine (EMDP); 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP); 3,4 methylenedioxyamphetamine (MDA); 3,4 methylenedioxymethamphetamine (MDMA); 3,4-dimethyl- methcathinone (DMMC); 3,4-methylenedioxy- *N*-ethylamphetamine (MDEA); 3,4-methylenedioxypyrovalerone (MDPV); 3′,4′-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP); 3′4′-methylenedioxy-α-dimethylamino-isovalerophenone (MDPBP); 4-ethyl-2,5 dimetoxiphenethylamine (2C-E); 4-Methylethcathinone (4-MEC); 4-fluoromethcathinone (4-FMC); 6-(2-aminopropyl)benzofuran (6-APB); 6-monoacetylmorphine (6-MAM); alpha-pyrrolidi- nohexiophenone (α-PHP); alpha-pyrrolidinopentiophenone (α-PVP); amphetamine (AMP); benzoylecgonine (BEG); cannabidiol (CBD); cannabinol (CBN); cocaethylene (CE); cocaine (COC); codeine (COD); Direct Immersion Solid-Phase Microextraction (DI-SPME); dispersive Solid-Phase Extraction (dSPE); Disposable Pipette Extraction tips (DPX); ecgonine methyl ester (EME); Headspace Solid-Phase Microextraction (HS-SPME); ketamine (KET); Lysergic acid diethylamide (LSD); mephedrone (4-MMC); methadone (MET); methamphetamine (MA); methylone (METH); Microextraction by Packed Sorbent (MEPS); morphine (MORP); *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine (MBDB); norketamine (NK); Solid Phase Extraction (SPE); Solid Phase Microextraction in Thin Film geometry (TF-SPME); Δ9-Tetrahydrocannabinol (THC).

Currently, the traditional methods of sample preparation, Solid Phase Extraction (SPE) and Liquid-Liquid Extraction (LLE), have been improved, with considerable advantagesand increasingly miniaturization. Therefore, innovative techniques are constantly being established for environment-friendly techniques that may combine with various analytical tools available in research laboratories worldwide [47,54].

All sample preparation methods must be investigated and optimized to guarantee a reliable and reproducible methodology [55]. Thus, within the topics below, every advantage and optimization by different sample preparation methods described in the literature for druganalysis during the last five years are addressed (Tables 2 and 3).

Protein precipitation and enzymatic hydrolysis

Prior to sample preparation with SPE or LLE techniques, protein precipitation and/or enzymatic hydrolysis are occasionally performed. Protein precipitation may clean the samples, exclude possible interferences and release the psychoactive substances that would be bound to the proteins [56–58]. It may be undertaken with organic agents that promote precipitation under mild conditions. Research reported in the literature for drug analysis usually uses methanol as an organic solvent. With such a solvent, the technique has been applied to analyze drugs in samples such as hair [23,59–61], DBS [25,38,62], tooth [32], serum samples [63] and oral fluid [45].

Williams et al. used acetonitrile as a solvent for protein precipitation in oral fluid samples [64] and Pan et al used ethyl ether with borate buffer (pH 9.2) in a biological blood matrix [65]. Trana et al., used 70 μL M3® buffer solution and 500 μL acetone: acetonitrile (8:2, v/v) as a solvent for protein precipitation in blood samples [7]. Ou et al. optimized the pre-processing of the hair matrix, assessing methanol, 1 % formic acid in methanol, and sodium hydroxide as solvents for protein precipitation [23]. Pure methanol solvent proved to have the highest efficacy and efficiency, linear correlation, and stable recovery rates of the methamphetamine and amphetamine analytes. Methanol is also widely used for producing a clear supernatant, enabling direct introduction into the chromatographic system.

Bassotti et al performed the dilution of the oral fluid (OF) matrix with 200 μL of Internal Standards containing 15 different drugs labeled with stable isotopes (methanol solution) [45]. After the centrifugation (10 min at 14,000 × g), the supernatant is transferred in glass vials and then 2 μL were directly injected in LC–MS/MS system.

Enzymatic hydrolysis disrupts the connection between the molecule to be analyzed and endogenous cofactors from the biotransformation process. The samplés preparation may occur through specific or non-specific enzymatic hydrolysis. Some authors have reported the use of specific enzymes for the hydrolysis of drug-protein conjugates in urine samples, such as the use of the β-glucuronidaseenzyme [57,60,66]. Consequently, researchers performed only protein precipitation or enzymatic hydrolysis, whilst the supernatant is directly introduced into the chromatographic system.

On the other hand, there are cases in which no pre-preparation or sample preparation is done, or rather, only the matrix is diluted. Malaca et al. performed the dilution of the oral fluid (OF) matrix with 1.0 mL water, before agitation and centrifugation, for psychoactive drugs analysis, including drugs, such as amphetamines, cocaine, *Cannabis*, heroin and morphine, by Ultra-High-Performance Liquid Chromatography (UHPLC) – Mass Spectrometry/Mass Spectrometry (MS/MS) [67].

Table 4

Gas chromatography (GC) methods described in selected literature between 2015 and 2020 for quantification and identification of drugs in biological samples.

Analyte	Injection volume	Stationary phase	Carrier gas	Detection system	Derivatization/ mass range	Ref.
Synthetic cannabinoids	1 µL	HP-5MS UI (30 m × 250 µm id., 0,25 µm)	Helium (Gradient temperature)	MS	*m/z*: 144–352	[26]
AMP, MA, MDA, MDMA, KET, MET, 4-MMC and methcathinone	1 µL	DB-5MS capillary column (30 m × 0.32 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS	*m/z*: 40–250	[87]
KET and NK	–	HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS/MS	*m/z*: 115–184	[74]
AMP, MA, MDA, MDMA, MDEA, MBDB, THC, MET, COC and CE	Desorption from fiber to GC	ME siloxane fused silica capillary column (0.33 µm × 12.5 m × 0.20 mm i.d.)	Helium (Gradient temperature)	MS	Derivatization with MSTFA 70 eV *m/z*: 55 – 390	[27]
AMP, MA, MDA, MDMA, MET, KET and 4-MMC	1 µL	HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS	70 eV *m/z*: 40–300	[86]
AMP, MA, MDA, MDMA, MDEA, MORP, 6-MAM, COD, MET, COC, EME, BEG, THC-COOH, antidepressants, benzodiazepines, antipychotics, and anticholinergic.	1 µL	Fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS	Derivatization with BSTFA and TMCS 70 eV *m/z*: 80 – 450	[59]
COC and MDMA	25 µL	DB-1 ms column (30 m × 0.25 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS	70 eV *m/z*: 50–450	[104]
MDPV	1 µL	Macherey-Nagel Optima 5MS Accent (30 m × 0.25 mm)	Helium (Gradient temperature)	MS	Derivatization with pyridine and acid anhydride 70 eV *m/z*: 44 – 600	[64]
MORP, 6-MAM, MET, EDDP and EMDP	1 µL	VF-5 MS (30 m × 0.25 mm i.d × 0.25 µm)	Helium (Gradient temperature)	MS	Derivatization with MSTFA *m/z*: 50 – 650	[71]
MORP	2 µL	Fused silica capillary column coated with HP-5 (30 m × 0.320 i.d. × 0.5 µm)	Nitrogen (Gradient temperature)	Nitrogen phosphorus detector (NPD)	–	[136]
MDMA, KET, THC and others	1 µL	Fused-silica capillary column Rxi-5Sil MS (30 m × 0.25 mm i.d., 0.25 µm	- (Gradient temperature)	MS/MS	Derivatization with TFA *m/z*: 50 – 650	[140]
AMP, MA, MDA, MDMA, MBDB and MDEA	2 µL	5 % de phenylmethylsiloxane column (30 m × 0.25 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS	Derivatization with MBTFA *m/z*: 90–180	[128]
MORP, 6-MAM and COD	2 µL	Capillary column of fused silica with 5 % phenylmethylsiloxane (HP-5 MS) (30 m × 0.25 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS/MS	Derivatization with MSTFA with 5 % TMS	[127]
THC, CBD, CBN and others syntethics cannabinoids	Online desorption	DB-5 (5 % phenylmethylsilicone) capillary column (30 m × 0.25 mm i.d × 0.25 µm)	Helium (Gradient temperature)	MS	*m/z*: 127–371	[44]
MORP, 6-MAM, MET and BEG	1 µL	Fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS	Derivatization with BFTSA 1 %TMCS *m/z*: 40–550	[36]
COC, AMP, synthetic cannabinoids, opiates and others	1 µL	TG-5SILMS column (30 m × 0.25 mm i. d. × 0.25 µm)	Helium (Gradient temperature)	HRMS	Positive ionization *m/z*: 40 – 650 (Resolution = 60,000 FWHM)	[65]
6-APB, METH, and others NPS	–	DB-5 ms column (30 m × 0.32 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS	Derivatization with PFPA: ethyl acetate (2:1)	[98]
GHB	1 µL	DB-5MS capillary column (30 m × 0.32 mm i.d. × 0.25 µm)	Helium (Gradient temperature)	MS/MS	Derivatization with BSTFA *m/z*: 40–500	[88]

where: 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH); 2,2,3,3,3-Pentafluoropropyl acrylate (PFPA); 2-ethyl-5-methyl-3,3 diphenylpyrrolidine (EMDP); 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP);3,4 methylenedioxyamphetamine (MDA), 3,4 methylenedioxymethamphetamine (MDMA); 3,4-methylene-dioxy- *N*-ethylamphetamine (MDEA); 3,4-methylenedioxypyrovalerone (MDPV); 6-(2aminoprppyl)benzofuran (6-APB); 6-monoacetylmorphine (6-MAM); amphetamine (AMP); benzoylecgonine (BEG); cannabidiol (CBD); cannabinol (CBN); cocaethylene (CE); cocaine (COC); codeine (COD); ecgonine methyl ester (EME); Gamahidroxibutirato (GHB); Gas chromatography (GC); High resolution mass spectrometry (HRMS); ketamine (KET); Mass spectrometry (MS); mephedrone (4-MMC); methadone (MET); methamphetamine (MA); methylone (METH); morphine (MORP); Nitrogen phosphorus detector (NPD); *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine (MBDB);*N*-methyl-bistrifluoroacetamide (MBTFA); *N*-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA); N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA); norketamine (NK); tandem mass spectrometry *(MS/MS);*Δ9-Tetrahydrocannabinol (THC); Tetramethylsilane (TMS)**;** trifluoroacetic acid (TFA); Trimethylchlorosilane (TMCS).

Liquid-liquid extraction

Liquid-liquid extraction, together with miniaturized descendants (Table 2), occurred in 20 out of 71 79 articles, or rather, 28.21 %, published between 2015 and 2020 and included in this review, that analyzed drugs. The main biological samples analyzed by LLE were the whole blood and its derivatives, others (dental biofilm, nails, bones and meconium), dry samples and urine (Fig. 2-A). Dry samples were exclusively prepared by liquid extraction. The miniaturized techniques used more frequently samples of whole blood and its derivatives, hair and urine. Grapp et al., for example, performed two types of LLE in urine, with different proportions of solvents, to promote an alkaline and neutral extraction, each screening different analytes [68].

Liquid extraction has also been applied to other matrices, such as DBS [37,69,70], bones [33] and fluid oral [26]. As shown in Table 2, in these cases, acetonitrile, methanol, ammonium hydroxide, hexane and ethyl acetate were the extraction solvents in LLE.

There are techniques in which the preparation of samples involves two extraction methods. This usually occurs when the matrix is complex, such as the nail. Preparation time, following method by Magalhaes et al. in which LLE precedes SPE, becomes more difficult. In this case, the nail hydrolysate isacomplex matrix with many interfering substances and high viscosity [71]. Consequently, prior to SPE, an acid LLE was performed to obtain cleaner extracts. LLE was evaluatedfor different extraction solvents (different proportions of *n*-hexane: ethyl acetate, chloroform: 2-propanol, among others) with best result 1 mL of methyl *tert*-butyl ether and 500 µL of 0.1 M phosphate buffer.

In a validated study for drug (morphine, cocaine, amphetamine, 6-acetylmorphine, MDA, MDMA, and others) determination in a dental biofilm matrix, the LLE technique was optimized by an alkaline extraction (borate buffer and 1-chlorobutane solvent) or extraction with different organic solvents (methanol, acetonitrile, acetone or dichloromethane) in an ultrasonic bath or microwave oven. Alkaline LLE was eliminated since it occurred in lower areas when compared to extraction with acetonitrile. Acetone and methanol showed inefficient extraction, but acetonitrile and dichloromethane showed the same extraction efficiency. Since acetonitrile proved to have less health risk, it was chosen as solvent. Moreover, the ultrasonic bathwas the preferred apparatus, in spite of equal efficiency due to thermal stress for analytes in a microwave oven [31].

In the case of other optimizations performed in LLE sample preparation method, an analysis performed in dry oral fluid (DOF) determined the most appropriate proportion of each chosen solvent, opting for a chemometric tool, with a centroid design method, built with three solvents (methanol, acetonitrile and 14 mM ammonium acetate buffer). The tool was significant for the optimization of a new sample preparation method, with a reduction in the number of experiments and the study of a simultaneous variation of important extraction factors through significant data, with shorter time period, reagents, resources and costs [72–74]. Thus, according to chromatographic responses, such as analytes'peak area (cocaine, benzoylecgonine, cocaethylene, amphetamine and MDMA), performance, resolution, sensitivity and peak distortion, the ideal proportion of these solvents was chosen: 200 µL of acetonitrile, ammonium acetate buffer (14 mM) and methanol (55:35:10, v/v) (Table 2) [75].

Conventional samples have also been prepared with LLE technique.

Two assays by Wiergowski et al. [52] and Franco de Oliveira et al. [58] analyzed blood samples extracted with different solvents: ethyl acetate and acetonitrile: methanol (80:20, v/v), respectively. It has been observed that, in the most recent study [58], the sample volume and extracting solvents volume were significantly lower than in the previous one [52], as described in Table 2. Its advantage is an environmentally friendly technique and a lower and satisfactory sample volume for the analysis.

Miniaturization LLE

Over the years, the techniques of sample preparation are being perfected and resulting in miniaturized and/or automated techniques. Due to their economic and environmental importance, the miniaturized approaches are relevant, as their principles with concepts of green chemistry are characterized with the use of less toxic solvents, as well as the reduction of solvent/sample volumés [76–79].

The miniaturization of the techniques os sample preparation presupposes achieving satisfactory analytical results, since it combines other potential advantages, such as a simpler procedure, faster analysis, greater extraction performance and the possibility of automation [76,80–82].

Alternative LLE methods have been developed, such as Salting-out Assisted Liquid–Liquid Extraction (SALLE), Hollow Fiber Membrane Liquid-phase Microextraction (HF-LPME), Dispersive Liquid-Liquid Microextraction (DLLME), Ultrasound-Assisted-Low-Density Solvent - Dispersive Liquid-Liquid Microextraction (UA- LDS-DLLME), Pressurized Liquid Extraction (PLE), prior to DLLME and Hollow Fiber-Renewal Liquid Membrane extraction (HFRLM).

SALLE was applied with small sample amounts and low solvent volumes, besides the use of organic salts friendly to MS in meconium samples before drug analysis by LC-MS/MS [83]. This extraction procedure was very efficient, easy and fast, resulting in relatively clean extracts without the need for expensive SPE columns, reducing the cost.

The remarkable advantages of DLLME technique, a type of LPME first introduced by Rezaee et al. [84], are its easiness, speed and low cost, especially suitable for the preparation of biological samples [85]. There is a strong comparison between this preparation and SPE because it offers greater advantages, or rather, the amount of solvent, costand time were reduced, coupled to greater enrichment. This technique employs ternary solvent mixtures and uses small volumes of solvent and sample [49].

DLLME has been recently applied for drug analysis in several matrices, such as blood [42,86,87], urine [87] and hair [39,88]. Fisichella et al. developed a blood method that used low volumes of organic solvents, ensuring extraction efficiency with a satisfactory level of sensitivity of the tested analytes [42]. Consequently, the smallest possible volume of solvent (extractors and dispersers) was tested to obtain a cloudy solution, resulting in 100 µL chloroform (extractor) and 250 µL methanol (disperser), offering economic and environmental advantages.

In recent years, UA-LDS-DLLME has been focused as an alternative method for the pre-treatment of samples due to ease, speed and low solvent consumption [88]. It proved to be effective in eliminating the effect of the complex sample matrices [88,89]. UA-LDS-DLLME differs from DLLME by the addition of ultrasonic energy to assist in the

Table 5

Liquid chromatography (LC) methods described in selected literature between 2015 and 2020 for quantification and identification of drugs in biological samples.

Analyte	Injection volume	Separation system	Stationary phase	Mobile phase	Detection system	Derivatization/ mass range	Ref.
Opiates, cocainics, THC, amphetamine type substances, ketamine and norketamine	1 µL	UHPLC	BEH C18 column (2.1 × 50 mm,1.8 µm)	90 % 5 mM ammonium formiate in 0.1 % aqueous formic acid and 10 % acetonitrile (Gradient elution)	MS/MS	Negative ionization m/z: 40–400	[12]
methylone, butylone, mephedrone, 4 methylethcathinone and NPS	1 µL	UHPLC	BEH C18 column (2.1 × 50 mm,1.8 µm)	95 % 0.1 % aqueous formic acid and 5 % metanol (Gradient elution)	MS/MS	Negative ionization m/z: 40–400	[12]
GHB, GBL and GHB- glucuronide	1 µL	UHPLC	HSS T3 C18 column (2.1 × 150 mm, 1.8 µm)	95 % 0.1 % aqueous formic acid and 5 % metanol (Gradient elution)	MS/MS	Negative ionization m/z: 40–400	[11,12]
AMP, MDMA, KET, BZE, COC, MORP COD, 6-MAM, MET, THC and NPS	1 µL	UHPLC	HLB (4.6 × 20 mm, 5 µm)	12.5 mM ammonium formate pH 9.5 and acetonitrile (Gradient elution)	MS/MS	Positive ionization m/z: 50–500	[8]
BZE, COC, MORP, 6-MAM, NPS and others	1 µL	HPLC	HLB (4.6 × 20 mm, 5 µm)	ammonium formate solution pH 9.5 and acetonitrile (Gradient elution)	MS/MS	Positive ionization m/z: 50–500	[7]
Synthetic cannabinoids	10 µL	UHPLC	Kinetex Biphenyl 100A (100 × 2.1 mm, 2.6 µm)	ammonium formate 2 mM in water with 0.1 % formic acid and ammonium formate 2 mM in methanol/acetonitrile 50:50 (v/v) with 0.1 % formic acid (Gradient elution)	HRMS	Positive ionization m/z: 50–650	[26]
AMP, MA, MDA, MDMA, COC BZE, THC, THC-OH THC-COOH, EDDP MET	10 µL	UHPLC	HSS C18 column (2.1 mm × 150 mm, 1.8 µm)	0.1 % formic acid in acetonitrile and 5 mM ammonium formate pH 3.0 (Gradient elution)	MS/MS	Positive ionization m/z: 90–345	[25]
MORP,6-MAM and MET	20 µL	HPLC	Chiral alpha-1-acid glycoprotein analytical column (100 mm × 94.0 mm × 5 µm)	Acetonitrile/ammonium acetate buffer (10 mmol/L, pH 7.0, 22:78v/v) (Isocratic elution)	MicrOTOF-Q	Positive ionization m/z: 50–1000	[97]
COC, CE, BEG, EME, AMP, MA, MDA, MDMA, MET, LSD, KET, EDDP, MORP, 6-MAM, NK buprenorphine, and fentanyl and analogues), benzodiazepines, Z-compounds	–	UHPLC	Kinetex Biphenyl column (2.6 µm × 100 × 2.1 mm)	Water with 0.1 % HCOOH and MeOH with 0.1 % HCOOH (Gradient elution)	MS/MS	Positive ionization m/z: 50–250	[42]
MDPV	50 µL	UHPLC	BEH C18 column (100 mm × 2.1 mm × 1.7 µm)	Water and methanol (90:10, v/v) (Gradient elution)	MS/MS	Positive and negative ionization m/z: 50–1000	[146]
AMP, COD, MET, MORP and NPS	2 µL	HPLC	Luna PFP column (150 mm × 2 mm × 5 µm)	Water with 2 % HCOOH and 2.0 mmol/L ammonium formate and methanol with 0.1 % HCOOH (95:5, v/v) (Gradient elution)	MS/MS	Positive ionization m/z: 50–350	[31]
MORP, COD, 6-MAM, MET, EDDP, COC, BEG, THC, THC-OH, THC-COOH, AMP, MA, MDMA, MDA and MDPA	10 µL	UHPLC	Acquity UPLC HSS C18 column (2.1 mm × 150 mm × 1.8 µm)	Acetonitrile with 0.1 % HCOOH and 5 mM ammonium formate (5:95, v/v) (Gradient elution)	MS/MS	Positive ionization m/z: 90–350	[37]
AMP, MA, MDMA, MDEA, MDA, KET, NK, MORP, COD, COC, BEG, LSD, MET, 6-MAM, THCA, PMA, PMMA, PCP, 4-MMC, METH, 2C-B and NPS	50 µL	HPLC	ACE5 C18 column (250 mm × 4.6 mm × 5 µm)	5 % acetonitrile with 0.1 % HCOOH and 95 % acetonitrile with 0.1 % HCOOH (100:0, v/v) (Gradient elution)	MS/MS	Positive ionization m/z: 55–470	[135]
4-MMC, MDPV and 4-MEC	10 µL	HPLC	Zorbax C18 (2.1 mm × 50 mm × 3.5 µm)	Acetonitrile with 0.1 % HCOOH and water with 0.1 % HCOOH (2:98, v/v) (Gradient elution)	MS/MS	Positive ionization m/z: 130–280	[39]
AMP, MA, KET, NK, LSD, PMA, PMMA, MDA, MDMA, MDEA, MET, COC-OH, COD, MORP, 2C-B, PCP and benzodiazepines, barbiturates.	10 µL	HPLC	EC-C18 column (2.1 × 100 mm × 2.7 µm)	Water with 0.1 % acetic acid and methanol (98:2, v/v) (Gradient elution)	Ion booster-MS	Positive and negative ionization	[69]

Table 5 (*continued*)

Analyte	Injection volume	Separation system	Stationary phase	Mobile phase	Detection system	Derivatization/ mass range	Ref.
MORP, 6-MAM, MET, COC, CE, BEG, MDPV, EDDP, 4-MMC and METH	5 μL	UHPLC	ACQUITY BEH Shield RP18 column (2.1 mm × 100 mm × 1.7 μm)	Water with 0.1 % HCOOH and acetonitrile with 0.1 % HCOOH (90:10, v/v) (Gradient elution)	PDA	Wavelength range: 200 – 400 nm	[72]
MORP, COD, AMP and others	10 μL or online desorption	HPLC	TF Accucore PhenylHexyl (100 mm × 2.1 mm × 2.6 μm).	2 mM aqueous ammonium formate with 0.1 % HCOOH and 2 mM aqueous ammonium formate with acetonitrile:methanol with 0.1 % HCOOH and 2-propanol-acetonitrile (Gradient elution)	MS/MS	Positive and negative ionization *m/z*: 138–1780	[124]
2-MMC, 3-MMC and 4-MEC	10 μL	HPLC	Biphenyl analytical column (100 mm × 2.1 mm × 2.7 μm)	Water/ methanol with 0.1 % HCOOH and methanol with 0.1 % HCOOH (95:5, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 140–230	[63]
CBD, COC, COD, MDMA, heroin, LSD, and others	1 μL	Nano-LC	Reverse phase column (75 μm × 500 mm × 2 μm)	Water with 0.1 HCOOH and MeCN with 0.1 % HCOOH (95:5, v/v) (Gradient elution)	Nano-MS	Positive ionization *m/z*: 100–750	[143]
Opiates, COC, AMP, KET, cannabinoids, cathinones and others	10 μL	HPLC	RSLC 120 C18 (2.1 × 100 mm × 2.2 μm)	Water with 5 mM ammonium formate with 0.1 % HCOOH and methol/acetonitrile (100:0. v/v) (Gradient elution)	MS	Positive ionization *m/z*: 100–800	[60]
THC, 11-OH-THC and THC-COOH	2 μL	UHPLC	BEH C18 column (2.1 × 100 mm × 1.7 μm)	Water with 0.1 % HCOOH and acetonitrile with 0.1 % HCOOH (35:65, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 120–350	[30]
25B-NBOMe and 4-CMC	10 μL	HPLC	Ascentis Express C18 (100 × 4.6 mm × 2.7 μm)	Water with 0.01 % HCOOH and acetonitrile with 0.01 % HCOOH (80:20, v/v) (Gradient elution)	MS	Positive and negative ionization *m/z*: 50 – 1000	[52]
CBD, COC, COD, heroin, LSD, MDMA, MORP, nicotine and others	1 μL	Nano-LC	EASY-Spray PepMap C18 (75 μm × 150 mm × 3 μm)	Water with 0.1 % HCOOH and MeCN with 0.1 % HCOOH (96:4, v/v) (Gradient elution)	Nano-MS	Positive ionization *m/z*: 170–1000	[137]
MORP, 6-MAM, COD and others	4 μL	HPLC	SB-C18 column (2.1 × 100 mm × 2.7 μm)	5 mM ammonium formate in water with 0.1 % HCOOH and methanol with 0.1 % HCOOH (95:5, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 45–340	[43]
AMP, MA, MDA, MDMA, MDPV, COC, BEG, EME, MORP, COD, EDDP, MET, α-PVP and other NPS	1 μL	UHPLC	HSS C18 (2.1 × 150 mm × 1.8 μm)	5 mM ammonium formate and acetonitrile with 0.1 % HCOOH (87:13, v/v) (Gradient elution)	MS	Positive and negative ionization *m/z*: 50 – 1000	[21]
AMP, MA, MDMA, MDEA, MDA, COC, BEG, MORP, COD, 6-MAM	10 μL	HPLC	Luna PFP column (150 mm × 2 mm × 5 μm)	Ammonium formiate with 0.1 % HCOOH and methanol with 0.1 % HCOOH (95: 5, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 70–350	[32]
COC, MORP and others	–	UHPLC	C18 (2.1 mm × 50 mm × 1.6 μm)	Acetonitrile with 0.1 % HCOOH and 5 mM ammonium formate with 0.1 % HCOOH (5:95, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 100–525	[57]
AMP, MA, MDA, MDMA, MDEA, MORP, 6-MAM, COD, MET, COC, EME, BEG, THC-COOH, antidepressants, benzodiazepines, antipychotics, and anticholinergic.	–	UHPLC	Acquity BEH C18 column (150 × 2.1 mm × 1.7 μm)	Water with 0.1 % HCOOH and methanol with 0.1 % HCOOH (70:30, v/v) (Gradient elution)	MS/MS	Positive and negative ionization *m/z*: 55–330	[33]
	10 μL	UHPLC			MS/MS		

Table 5 (*continued*)

Analyte	Injection volume	Separation system	Stationary phase	Mobile phase	Detection system	Derivatization/ mass range	Ref.
AMP, MA, BEG, EDDP, MDA, MDMA, MET, COD, MORP and COC			Acquity UPLC® HSS T3 column (100 mm × 2.1 mm × 1.8 μm)	Acetonitrile/ 2 mM ammonium formate with 0.1 % HCOOH (95:5, v/v) (Gradient elution)		Positive ionization *m/z*: 80–340	[70]
AMP, MA, 6-MAM, MDA, MDMA, COC, NCOC, AEME, CE, COD, MORP, THC-COOH and benzodiazepines	3 or 15 μL	HPLC	Raptor Biphenyl column (50 mm × 3 mm × 2.7 μm)	2 mM ammonium formate with 0.1 % and acetonitrile (92:8, v/v) or 0.2 % acetic acid in water and acetonitrile (65:35, v/v) (Gradient elution)	MS/MS	Positive and negative ionization *m/z*: 40–340	[58]
Synthetic opiods	20 μL	UHPLC	Zorbax Rx-SIL (3 mm × 100 mm × 1.8 μm)	Ammonium formiate and acetonitrile (10:90, v/v) (Isocratic elution)	MS/MS	Positive ionization *m/z*: 90 – 390	[145]
AMP, MORP, COC, COD, CBD, KET, MDMA, THC and others	2 μL	UHPLC	UPLC BEH C18 column (2.1 mm × 75 mm × 1.7 μm)	Water with 0.1 % HCOOH and acetonitrile with 0.1 % HCOOH (90:10, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 90–390	[67]
AMP, MA, MDMA, MDPV, α-PVP, 4-MMC, METH, butylone, flephedrone, and naphyrone	–	UHPLC	EclipsePlus C18 (50 mm × 2.1 mm × 1.8 μm)	Water with 0.1 %HCOOH / 10 mM ammonium formate and Methanol with with 0.1 % HCOOH / 10 mM ammonium formate (90:10,v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 90–290	[83]
Opioids	–	HPLC	Poroshell 120 EC-C18 column (2.1 × 100 mm x 2.7 μm)	Water with 0.1 % HCOOH and acetonitrile with 0.1 % HCOOH (90:10, v/v) (Gradient elution)	MS	Positive ionization *m/z*: 100–1000	[138]
Synthetic cannabinoids	1 μL	HPLC	Kinetex Biphenyl column (50 mm × 3 mm × 2.6 μm)	Water with 0.1 % HCOOH and acetonitrile (45: 55, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 90–460	[64]
COC and metabolites	1 μL	UHPLC	Acquity HSS C18 column (150 × 2.1 mm × 1.8 μm),	Ammonium formate 5 mM (pH 3) and acetonitrile containing 0.01 % HCOOH (95:5, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 82–322	[38]
COC, MORP, CBD, THC, MET, MDMA, and others	6 μL	HPLC	Kinetex PFP (100 mm × 2.1 mm × 2.6 μm)	Water and acetonitrile/ methanol, 50/50 (v/v) acidified with 0.1 % HCOOH (100:0, v/v) (Gradient elution)	HRMS	Positive and negative ionization *m/z*: 50–500	[49]
MDMA, AMP, COC-OH, CE and BEG	20 μL	HPLC	Phenomenex Kinetex HILIC column (150 × 4.6 mm × 2.6 μm)	Acetonitrile, ammonium acetate buffer 14 mM, and methanol (55:35:10 v/v) (Isocratic elution)	MS	Positive ionization *m/z*: 75–318	[75]
AMP, COC, opioids, THC-COOH and others	10 μL	HPLC	Phenomenex C18 (2.1 × 50 mm × 2.6 μm)	Ammonium formate 10 mM pH 3.3 and methanol with 0.1 % HCOOH (2:98, v/v) (Gradient elution)	HRMS	Positive and negative ionization	[62]
COC, MORP, KET and others.	10 μL	HPLC	Poroshell 120 EC-C18 analytical column (3.0 × 50 mm × 2.7 μm)	5 mM ammonium formate with 0.1 % HCOOH in water and 0.1 % HCOOH in acetonitrile (85:5, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 40–450	[66]
MORP, 6-MAM, MET, BEG, COC, CE, AMP, EDDP, MDA, MDEA, MDMA, MA, KET, LSD and COD	10 μL	UHPLC	Acquity UPLC HSS T3 (2.1 mm. × 100 mm × 1.8 μm)	0.1 % HCOOH in water and methanol (90:10, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 40–380	[48]
6-MAM, MORP, COD, AMP, MA, MDA, MDMA, COC, BEG, CE, EME, KET, NK, THC-COOH and and other	1 μL	HPLC	Biphenyl columns (50.0 × 3.0 mm × 2.7 μm)	Methanol and 10.0 mM ammonium formate (pH 3.0) (3:97, v/v) (Gradient elution)	MS/MS	Positive and negative ionization *m/z*: 41–387	[51]
COC, BEG, CE, COC-OH	–	HPLC	Shim-pack XR-ODS II column (75 × 2.0 mm × 2.2 μm)	0.1 % HCOOH in water and acetonitrile (100:0, v/v) (Gradient elution)	MS	Positive ionization *m/z*: 50–350	[47]
COC, MDMA, Nicotine, Naloxone, COD and others	Inline desorption	HPLC	Thermo Scientific™ Cyclone-P TurboFlow™ column (0.5 × 50 mm) and Kinetex Biphenyl (50 × 2.1 mm)	Water containing 10 mM ammonium formate and methanol containing 10 mM ammonium formate (100:0, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 58–470	[133]
AMP, MA	1 μL	UHPLC	Poroshell 120 PFP column (2.1 × 100 mm	Acetonitrile and aqueous buffer solution containing 20	MS/MS	Positive ionization *m/z*: 65–168	[23]

(*continued on next page*)

Table 5 (*continued*)

Analyte	Injection volume	Separation system	Stationary phase	Mobile phase	Detection system	Derivatization/ mass range	Ref.
			× 1.9 μm)	mmol/L ammonium acetate and 0.1 % HCOOH (70:30, v/v) (Gradient elution)			
NPS	5 μL	UHPLC	Zorbax Eclipse Plus C18 RRHD column (2.1 mm × 100 mm × 1.8 μm)	2 mM ammonium formate/ 0.2 % HCOOH in water and 2 mM ammonium formate/0.2 % HCOOH in methanol (Gradient elution)	MS/MS	Positive ionization *m/z*: 100–388	[61]
Synthetic Opioids	5 μL	UHPLC	Acquity UPLC BEH phenyl column (2.1 × 100 mm × 1.7 μm)	% HCOOH in water and methanol with 0.1 % HCOOH (70:30, v/v) (Gradient elution)	MS/MS	Positive ionization *m/z*: 84–417	[100]
COC, BEG, CE, Δ-9-THC, 6-MAM, MORP, COD, MET, EDDP, AMP, MA, MDMA, MDA, MDE, KET and others	2 μL	UHPLC	Hypersil PFP Gold column (50 × 2.1 mm, 1.9 μm particle size)	0.1 % formic acid and 50 % methanol/50 % acetonitrile with 0.1 % formic acid (95:5, v/v). (Gradient elution)	MS/MS	Positive ionization *m/z*: 120–470	[45]
THC-COOH	20 μL	HPLC	Halo C18 column (2.1 mm × 75 mm, 2.7 μm)	0.5 mM ammonium formate and 0.5 mM ammonium formate in acetonitrile (95:5, v/v) (Gradiente elution)	MS/MS	Negative ionization *m/z*: 245–346	[129]

where: 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH); 4-methoxyamphetamine (PMA); (1-(4-chlorophenyl)-2-(methylamino)-1-propanone) (4-CMC); 11-hydroxy-tetrahydrocannabinol (11-OH-THC); 11-nor-9-carboxy-Δ⁹-tetrahydrocannabinol (THC-COOH); 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP); 3,4 methylenedioxyamphetamine (MDA); 3,4-Methylenedioxy-*N*-ethylamphet-amine (MDE); 3,4 methylenedioxymethamphetamine (MDMA); 3,4-methylenedioxy- *N*-ethylamphetamine (MDEA); 3,4-methylenedioxy-*N*-propyl-amphetamine (MDPA); 3,4-methylenedioxypyrovalerone (MDPV); 4-Bromo-2,5-dimethoxyphenethyl-amine (2C-B); 4-Methylethcathinone (4-MEC); 6-monoacetylmorphine (6-MAM); alpha-pyrrolidinopentiophenone (α-PVP); amphetamine (AMP); Anhydroecgonine methyl ester (AEME); benzoylecgonine (BEG); buphedrone (MABP); cannabidiol (CBD); cocaethylene (CE); cocaine (COC); codeine (COD);, delta-9-tetrahydrocannabinol (Δ-9-THC); ecgonine methyl ester (EME); Gama-hidroxibutirato (GHB); Gama-butirolactona (GBL); High performance liquid chromatography (HPLC); High resolution mass spectrometry (HRMS);hydroxy–cocaine (COC-OH); ketamine (KET); Liquid chromatography (LC); Lysergic acid diethylamide (LSD); nanoscale liquid chromatography (Nano-LC); Mass spectrometry (MS);mephedrone (4-MMC); methadone (MET); methamphetamine (MA); methylone (METH); morphine (MORP); norbuprenorphine (*N*-BUP); norcocaine (NCOC); norketamine (NK); *para*-methoxymethamphetamine (PMMA); phencyclidine (PCP); Photodiode Array (PDA); Supercritical Fluid Extraction (SFE); tandem mass spectrometry (MS/MS); tetrahydrocannabinol carboxylic acid (THCA); Δ9-Tetrahydrocannabinol (THC); Ultra-high performance liquid chromatography (UHPLC);
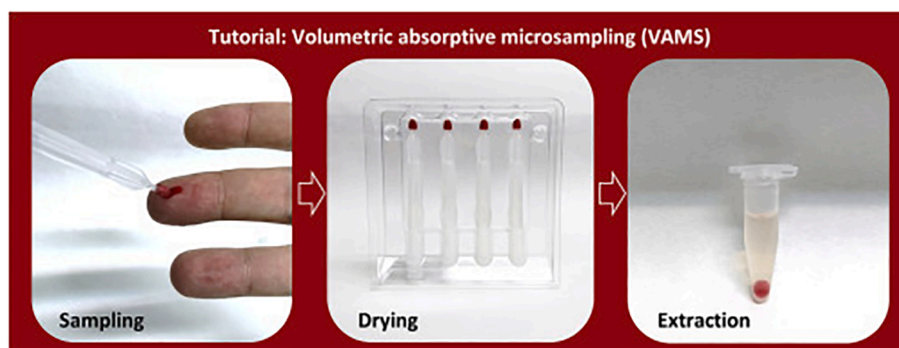

Fig. 1. Volumetric absorptive microsampling (VAMS). In capillary blood VAMS sampling, a blood drop is obtained by finger pricking and then sampled by touching the blood surface with the VAMS device. After sampling, multiple VAMSs were enclosed in the dedicated clamshell to drying step. In the end, sampled VAMS tip detached from the handle, in a microtube containing extraction solvent. Reference: 41.

emulsification process without any dispersing solvent, preventing the loss of the extraction solvent and significantly reducing the use of organic solvent, and thus an improvement in extraction efficiency. Further, the extraction solution of a lower density than water is easily collected after demulsification. It eliminates the effect of the complex sample matrix and becomes suitable as a biological sample for drug analysis [87].

HF-LPME is one of the most promising techniques for sample preparation for pre-concentration, separation, and cleaning purposes, particularly in complex matrices. The disposable nature of the hollow fiber, used in this technique, excludes the possibility of sample transfer and guarantees high reproducibility. Moreover, the pores in the hollow fiber walls are filled with solvent, ensuring selectivity for this technique since it prevents the extraction of materials with high molecular weight [47,90–92].

In 2015, Meng et al. [87] carried out a comparison between the two sample preparations, UA-LDS-DLLME and HF-LPME, and improved them with regard to the selection of the extraction solvent and the pH of the sample solution, blood and urine. The authors concluded that the extraction's highest efficiency rate was obtained by DLLME when compared to HF-LPME, despite the greater reproducibility and precision rate of HF-LPME. The above could be explained by DLLME corresponding to the much larger surface areas between extraction and sample solutions. Since adulterations by DLLME were also easily co-
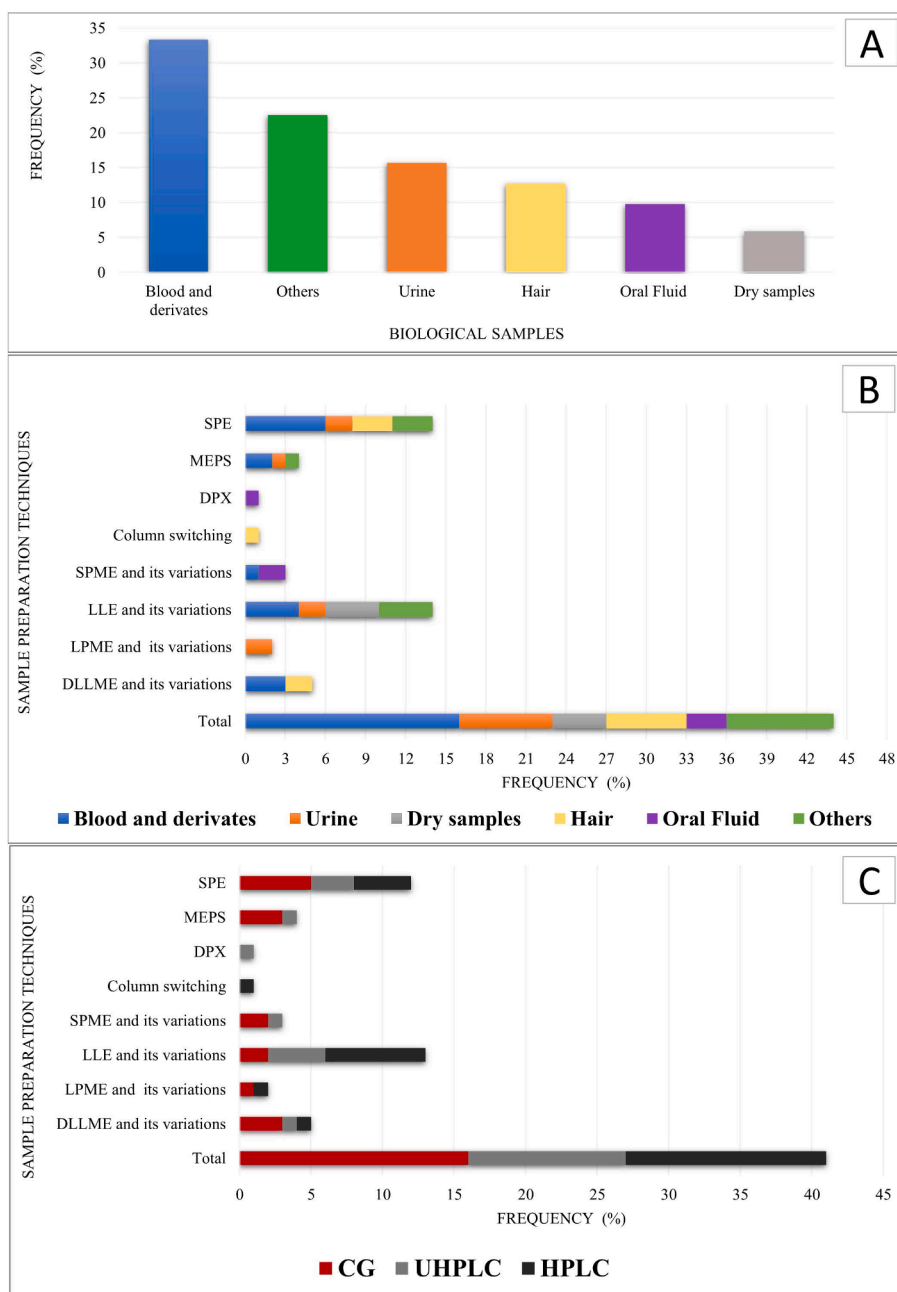
Fig. 2. Correlation graphs of biological samples, technique of sample preparation and chromatographic methods used in the selected articles. Where: A: Frequency (%) versus biological samples; B: Techniques of sample preparation versus frequency (%) according to biological samples, C: Techniques of sample preparation versus frequency (%) according to chromatographic methods.

extracted by the tiny droplets of the organic extractor, it resulted in worse repeatability, especially in more complex samples, such as blood [86].

The methodology described by Meng et al. [87] has been contested due to the large volume of blood sample used in DLLME (1 mL) when compared to 200 μL by Lin et al. [86]. The latter authors, who used methanol as a solvent for protein precipitation, also employed it as a dispersant solvent of Ultrasound-Assisted -Dispersive Liquid-Liquid Microextraction (UA-DLLME) technique. Lin et al. also optimized the UA-DLLME method through different solvents [86]. In fact, methanol had the best emulsification performance, followed by ethanol, iso-propanol and acetone. Results indicated that there was probably a strong relationship between emulsification and extraction efficiency. On the other hand, the extraction solvent was dichloromethane, revealing

greater extraction efficiency, and showed a better performance than toluene, which was used by Meng et al. [87]. Further, pH 13 was chosen due to a greater efficiency in the extraction of analytes, whilst ultra-sound time (2 min) was similar to that in previous study (3 min) [87]. For most of the drugs analyzed, the limit of detection (LOD) in the two methods was lower when UA-LDS-DLLME-GC–MS was used [87], varying from 1 to 4 ng mL^{-1} when compared to 10 ng mL^{-1} for LOD in all analytes employing UA-DLLME-GC–MS [86].

Owing to HF-LPME versatility, some modified approaches may be performed, such as Hollow Fiber-Renewal Liquid Membrane extraction (HFRLM). In this technique, an additional amount of extraction solvent is addeddirectly to the sample with the supported liquid membrane (SLM), composed of a hydrophobic hollow fiber with organic solvent. This approach provides a high relationship among the organic/aqueous
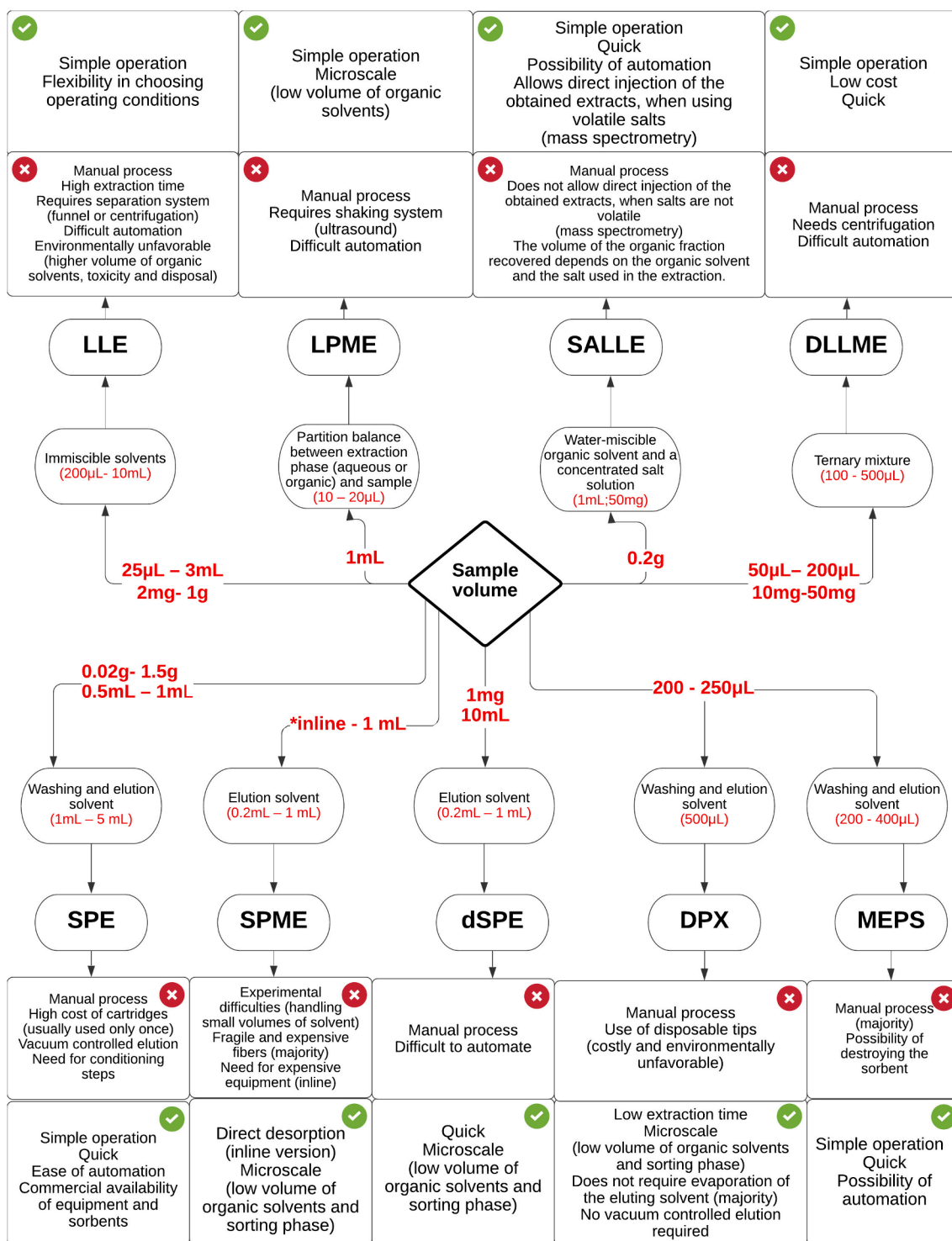
Fig. 3. Advantages and disadvantages of the main sample preparation techniques published between 2015 and 2020.

phases, whilst the microplots constantly renew the SLM solvent film, increasing mass transfer rate [93,94]. Further, HFRLM allows the automation of the analysis, which may also be achieved by using other techniques, such as SPME or SPE [47].

The HF-LPME method was applied to a diluted urine sample. So that extraction may be optimized, univariate and multivariate planning (simple central composite design and Doehlert matrix) was carried out and parameters, such as solvents, extraction time, ionic strength and desorption time, were assessed [47].

PLE-DLLME and UA-LSD-DLLME, two alternative methods to LLE

[59,91] were analyzed for the preparation of hair samples for drug analysis. Vincenti et al. [49] evaluated the performance of PLE – DLLME to two different extractions (digestion with sodium hydroxide and digestion with methanol), common in the literature, for comparative purposes [95,96]. Results showed that the extraction of certain analytes was effective for alkaline digestion (amphetamine, ketamine and synthetic cannabinoids), whilst, under the same conditions, others were unstable (cocaine, benzoylecgonine, morphine and methadone). On the other hand, methanol extraction was satisfactory for substances such as synthetic cannabinoids, cationates and ketamine. However, an increase

in the matrix effect due to the lack of extract cleaning was reported. Posterior to optimization, PLE-DLLME method revealed comparable results concerning the other two extractions for the detected substances, proving effectiveness in the application in the hair matrix. PLE guaranteed an automated process, coupling decontamination, drying and extraction of substances in the hair matrix in a short time (10 min) [49]. DLLME optimizations for the adequation of operational conditions in the wake of the large number of analyzed analytes were: four extracting solvents were tested (chloroform with the best result), dispersing solvents (isopropanol), volume and extraction time (200 μL and 10 min), pH effect (pH 11), *salting-out* effect (addition of NaCl). PLE and DLLME extraction solvents and best results of the cleaning technique were the same. In the case of pH evaluation, rate 3.5 was the most appropriate (although some analytes prevail at pH 7). In spite of low recoveries (benzoylecgonine and morphine), when compared to the literature (methanol) [96], the greatest loss occurred due to cleaning (DLLME). However, this cleaning was efficient for a wide range of substances, and others that may be implemented in a single analysis, with a decrease in time and costs. DLLME also guaranteed clean extracts, an enrichment factor that contributed to themethod's sensitivity and robustness.

The UA-LSD-DLLME method, used by Meng et al., employed a homogenizer device with high-frequency vibration for the first time in hair samples, associated with DLLME. DLLME optimizations evaluated 7 different extraction solvents (ethyl acetate had the best efficiency), the effect of the samplés pH (4.3) and effect of the extractiońs solvent volume (180 μL) [88].

Solid-phase extraction

SPE technique, new formats and miniaturizations (Table 3) were reported in 22 out of the 79 papers published between 2015 and 2020, or rather, 31.02 % of total studies included in this review. The main biological samples analyzed by SPE were the whole blood and its derivatives, hair, others (sweat, tissue specimens, nails and bone) and urine (Fig. 2-A). The miniaturized techniques used more frequently samples of oral fluid, whole blood and its derivatives, hair, urine and others.

In its classic cartridge mode, the technique is not only widely used but advantageous due to clean extracts [83]. It has been applied in blood matrix [30,51,97,98], urine [30], serum [68], *postmortem* fluids and tissues (vitreous humor, liver, lung, kidney, spleen, muscle, brain, heart, and bile) [30], nails [11,12,71] and bones [36].

In the highly complex matrix of hair samples, SPE has also been used [12,99,100], albeit with disadvantages, namely, a long, costly and complex procedure, featuring a great amount of adsorbent, solvent and sample.

Due to these inconveniences, new SPE formats have emerged which have prioritized the miniaturization of the technique [83,101], such as Dispersive - Solid Phase Extraction (d-SPE), also applied in hair matrix. The d-SPE is based on the addition of an adsorbent directly to the analytical solution, followed by dispersion, favoring the contact between adsorbent and analytes [102,103]. It is a specialized technique for high-efficiency cleaning accompanied by reduced loss of several analytes when compared to SPE. Further, it is advantageous due to decrease in total purification time, costs and labor [48].

Freni et al. [99] performed d-SPE extraction with hydrochloric acid, whilst in two other works extraction was performed with methanol, prior to cleaning [48,100]. Shin et al. optimized the effect of 4 extraction solvents, namely: methanol (chosen due to the stability of the 75 analytes analyzed after extraction), acetonitrile, methanol in 10 mM formic acid (5:5, v/v; pH 5.0) and methanol in 10 mM ammonium formate (5:5, v/v; pH 7.0) [48]. Researchers also optimized the cleaning stage, comparing d-SPE with simple filtration, SPE (Hybrid SPE-30 mg cartridge) and LLE (methyl *tert*-butyl ether – dichloromethane (5:5, v/v).

Ferrari et al.[104] also optimized the preparation of a blood sample by d-SPE for drug concentration and analysis by GC–MS [66]. The authors also compared this technique with Solid Liquid Extraction-Low Temperature Partitioning (SLE-LTP). The SLE-LTP consists of adding an extracting solvent to the solid sample, which may be mixed in water but less dense, albeit still liquid at −20 °C. The mixture is then stirred and taken to the freezer (−20 °C) for a period of ≥ 3 h. At this stage, the mixture in the aqueous phase and the sample are frozen. Analytes migrate to the organicphase, which is isolated and analyzed [105]. The d-SPE with acetonitrile was used for validation, according to the lower interference of endogenous compounds in the matrix and higher recovery performance for most analytes [104].

Sorribes-Soriano et al. [101] developed a sample preparation technique, named Pipette tip-based poly (methacrylic acid-*co*-ethylene glycoldimethacrylate) monolith, for extraction, for the concentration of analytes (cocaine, amphetamines, among others) in oral fluid matrix and analysis by UHPLC-MS-MS [54]. Techniqués advantages comprise ease in its use, miniaturization of the system, reduced amount of sample, reduced solvent consumption and high sample efficiency using multichannel portable pipettes or robotic liquid handling systems [105]. Although commercial silica-based monolithic pipettes exist, they have their disadvantages when compared to a post-treatment step that needs to be performed [106]. The study aimed at synthesizing a monolithic polymer (Table 3) to perform the Disposable Pipette Extraction (DPX) technique [101].

Miniaturization SPE

Among the advances achieved by the miniaturization SPE of conventional sample preparation techniques are the synthesis of new sorbent materials, their reuse and the use of small volumes of the sorting phase, in addition to other advantages shown in Fig. 3 [76,80,82].

The Solid Phase Microextraction (SPME), developed by Pawliszyn et al, similar to SPE, it is a sample preparation technique that combines extraction and pre-concentration of analytes in biological matrices [107]. Taken together, the extracts obtained do not require additional cleaning procedures. Thus, SPME is a relatively simple, fast and sensitive technique [107–109].

The extraction of the analyte by SPME can be carried out by a fused silica capillary or extraction phase packed in a stainless-steel support. The non-selectivity of the phase provides the extraction and pre-concentration of a wide range of small molecules that are desorbed, according to their physical and chemical properties, and interfere with chromatographic analysis [109].

SPE is mainly used for the extraction of organic compounds in aqueous samples in which the analytes are adsorbed directly from the sample to the fiber. Consequently, the analyte partition of the matrix in the stationary phase occurs until equilibrium is reached. Most automated SPME equipment is concentrated in SPME categories, such as Direct Immersion - Solid Phase Microextraction (DI-SPME) [110,111], Headspace-solid phase microextraction (HS-SPME) [112–114] or Thin-Film Solid Phase Microextraction (TF-SPME), being the sample, extracted by these methods, injected directly into GC. TF-SPME favors the execution of a combination between sampling (*in situ*) and sample preparation by direct immersion. Technique becomes more advantageous due to speed, decreasing errors and elimination of risks due to analytes' instability, since it is performed in a single step. This is possible due to the small size and biocompatibility of the SPME coating, enabling *in vivo* sampling by direct immersion extraction mode [108,115,116].

TF-SPME, a new SPME geometry, provides greater sensitivity and shorter extraction time. Bessoneau et al. performed the sampling (*in situ*) coupled to sample preparation by TF-SPME in saliva samples [109]. The authors evaluated two types of the membrane: balanced lipophilic and Hydrophilic Lipophilic Balance (HLB) and octadecyl column (C18). TF-SPME was applied to saliva *in vivo* and *ex vivo* for comparison. In both cases, the analytes (cannabinoids, heroin, codeine, and others) were extracted using immobilized membranes by polyacrylonitrile (HLB-PAN) and polydimethylsiloxane (HLB – PDMS) and analyzed by

chromatographic techniques, LC-MS/MS and GC–MS, respectively.

Anzilloti et al. performed an *in line* DI-SPME in an oral fluid matrix and compared it to HS-SPME [44]. For both sample preparations, the most appropriate capillary coatingwas optimized, including poly-dimethylsiloxane (PDMS), polyacrylate (PA), and poly-dimethylsiloxane/divinylbenzene (PDMS/DVB). PDMS was selected. DI-SPME-GC–MS was more effective for the analysis of the drugs. Analysis with HS-SPME-GC–MS could detect successfully Δ^9 –THC, cannabidiol (CBD) and cannabinol (CBN) only. Consequently, DI-SPME has shown several advantages, such as simplicity, speed, low cost, and easy appli-cability. It is a valid alternative to the conventional extraction of sample preparation [117,118].

Gentili et al. performed the application of HS-SPME in sweat sam-ples, with capillary PDMS (100 μm) [27]. Prior to SPME, a pre-preparation was performed to extract the possible adsorbent system pad analytes from the mobile device with 200 μL of hydrochloric acid (HCl) (1 M) for 60 min, at 60 °C, and 200 mg of K_2CO (after cooling).

Finally, another variation of SPE miniaturization is extant, namely, Microextraction by Packed Sorbent (MEPS). The technique comprises easy, fast, and resistantecological procedure (low sample volumes and solvents). Absorbent may be reused for>90 times. MEPS columns have the same adsorbents as those of the SPE column, being suitable for most biological samples [87,110,119–126].

SPME has a higher degradation risk in its system when compared to MEPS. The number of extractions is smaller and even longer, with smaller recoveries resulting from the analytes. Consequently, MEPS is a more sensitive technique, particularly for the most complex samples [110,127].

MEPS technique requires care to preserve the extractor adsorbent and to avoid damage to the sorption phase and to the extraction process itself. Particularly in the case of matrices such as blood and plasma, dilution is necessaryto reduce viscosity, avoid coagulation and/or block the adsorbent, obtaining low back pressure [110]. All studies with sample preparation by MEPS performed dilution of the samples (blood, plasma, and urine) prior to the technique [67,72,74,127].

Malaca et al. developed several tests to optimize procedures for extracting amphetamine in urine by MEPS [128]. MEPS cartridges were tested by reverse phase (C18) and M1 (C8 plus SCX). Extraction with SCX cartridge was not detectable for tested analytes (amphetamines, methamphetamines and other drugs). Ammonium acetate pH 6.7 (0.1 M), at 100 μL, was employed for dilution of urine, aimed at reducing matrixviscosity, preserving pH and the ionization of all analytes. It guaranteed a greater relative peak for the analytes analyzed by GC–MS. In the case of washing the adsorbent, two solutions (water and 5 % methanol), replacing acetic acid, were chosen for optimizations, as they guaranteed better removal of interferents, better affinity of compounds to the adsorbent, and prevention of loss of target analytes. A larger volume (150 μL) of the tested wash ensured greater recoveries and chromatograms with less interference. In the case of the number of sample extraction cycles, there was an increase in analytes recovery as cycles increased. Consequently, the use of nine sorptions/dissorptions of the sample was defined. Finally, there were four elution cycles due to greater recoveries, coupled to 2 % of ammonium hydroxide, ensuring greater extraction efficiency. Optimizations were carried out in a multivariate mode, evaluating the highest extraction efficiency for most analytes.

Fernández et al. used a chemometric tool to assess the extraction factors that influence MEPS procedure, in plasma, for the analysis of synthetic cationates, cocaine andbiotransformation products by UPLC-PDA [72]. Prior to application in MEPS, methanol (200 μL) was added for protein precipitation and removal of endogenous compounds, after which the supernatant was diluted in phosphate buffer (pH 8). Alkaline was preferable since the analytes studied (pKa ≈ 8) were in ionic solu-tion. The ternary eluting solvents (dichloromethane: 2-propanol: ammonium hydroxide) in different proportions were efficient for most analytes, albeit with unsatisfactory results when pure or biphasic. The

use of ultrapure water, acetic acid, methanol and formic acid also resulted in analytes eluted in cleaning. Consequently, the washing so-lution was a mixture of water: methanol (90:10, v/v).

Factors assessed by Fernández et al. were selected as the best for sample preparation: sample volume (300 μL), drying time (0.5 min), number of sample extraction cycles (10) and volume of elution (200 μL) [72]. Adsorbent was M1 (C8/SCX) in a mixed mode, although, in the case of morphine and methadone, responses were slightly better in the C18 phase. Highest washing volume rate (200 μL) resulted in better extractions, except for morphine (50 μL), with 150 μLas average. The above was highly promising for most of the analyzed analytes (morphine, cocaine, methadone, methylone, benzoylecgonine, cocaine, cocaethylene, MDPV, EDDP, 4-MMC, METH). In the methodology by Prata et al., after protein precipitation in a blood sample, the extraction factors of the samplés preparation procedure by MEPS, for analysis of codeine, morphine, and heroin, by CG-MS/MS, were optimized [127]. An experiment chemometric design was carried out with 20 sample extraction cycles, 1 solvent washing cycle, 3.36 % formic acid in the solution wash and 11 elution solution cycles and 2.36 % NH_4OH. Results were obtained from a factorial surface response methodology (RSM) by Minitab.

Moreno et al. used a multivariate approach to assess the factors that may influence MEPS procedure for ketamine and norketamineanalysis in plasma and urine [74]. The factors evaluated, such as number of sample extraction cycles, percentage of acetic acid for activating the adsorbent, percentage of methanol in cleaning and percentage of ammonia in the elution solvent, provided results evaluated by Pareto graphs. The only factors with a significant response were the percentage of methanol in the washing stage for norketamine in urine (15 %) and the number of extraction cycles (26) for the two analytes, in the plasma. The number of extraction cycles in plasma was greater than in urine (8 cycles). In fact, plasma matrix should be diluted 20 times to prevent obstructing the adsorbent. The 26 cycles would be necessary to allow the entire sample to pass through the mixed adsorbent M1 (C8 and SCX). Other factors, such as acetic acid (5.25 % for urine and 0.1 % for plasma), methanol (15 % for urine and 10 % for plasma), and ammonia (6 % for urine and 3 % for plasma), were selected according to the best apparent response and their influence was studied by univariate approach.

Helfer et al. performed automated online extraction by turbulent flow chromatography (TurboFlow) to extract drugs and metabolitesfrom the urine matrix [124]. The technique was compared to other types of sample preparation, dilution (3 and 10 times), and precipitation. Tur-boFlow has already proved to be an advantageous technique when compared to off-line extraction techniques, such as precipitation, SPE or LLE, due to its fast and self-coupled process. The combination of the silica-based extraction column and phenylstyrene/styrene/divinylben-zene revealed a better extraction performance for many classes of drugs [125,126].

Cho et al. also developed an online extraction using a column switching method aiming eliminate the high background level of the hair matrix lipids produced from alkaline hydrolysis of the hair. After a pre preparation using sodium hydroxide by hair samples be digested and organic solvent for extraction, they realized direct introduction into the chromatography system. The column switching system was configured with three columns: pre-column, trap colunm and analytical column, all of them consisting of C18. The method showed be selective and sensitive for the identification of THC-COOH in human hair [129].

Online extraction showed lower and better detection limits when compared to preparation by manual dilution, and similar ones when compared to precipitation, like proved by Helfer et al. [125]. However, relatively high signal suppression was observed in online mode in the both previlosy described articles, with lower amounts being detected when the matrix was diluted. Consequently, preparation with Turbo-Flow or C18 did not completely remove the interferers from the matrix. Moreover, there was a carryover effect on online preparation and

precipitation. In contrast, the precision tend to be better because of the online preparation [125,129]. In general, the online technique was advantageous due to its low cost, miniaturization, ease and total processing time [125,129–132].

Analytical techniques

The identification of drugs of abuse in biological samples may be performed by targeted or non-targeted analyses. When there is a suspect substance or a specific number of substances to be analyzed, the targeted analysis is performed. However, when there is no targeting of the toxic agent, a non-targeted analysis is conducted. In these cases, the analytical strategy should ideally monitor many toxic agents simultaneously. In fact, Systematic Toxicological Analysis is employed by all laboratories [21].

Systematic Toxicological Analysis generally consists of a combination of analytical strategies usually divided into two stages: screening analysis and confirmation. Regardless of the approach adopted, the toxicological analysis concludes with precision and unequivocalness whether any drugs were used or not [51,63].

During many years, primary screening analyses comprised immunoassay techniques only [51,133]. Immunoassay methods are cost-effective, quick to perform, usually using small sample volumes and automated systems [57,133]. However, immunoassays have a lack of specificity, or rather, they cannot differentiate drugs of the same class. Several synthetic compounds are not detected and cross-reactions may also occur which may result in false-positive. Consequently, additional confirmatory analyses are required [57,87,104,133,134].

A study by Kahl et al. evaluated the use of an Enzyme Linked Immunosorbent Assay (ELISA) and compared it to the chromatographic method (LC–MS/MS) [66]. One hundred samples were analyzed by the two methods and the results showed that the LC–MS/MS was able to detect a greater number of substances in some samples, namely benzoylecgonine (26 %), lorazepam (33 %), and oxymorphone (60 %). The study also proved that the costs for carrying out the analysis of biological samples using the chromatographic technique were lower when compared to the immunological one. However, the cost of purchasing and preserving the instruments has not been computed.

Lee et al. had already carried out a study comparing LC-MS/MS and immunoassay techniques [135]. When analyzing 779 urine samples by the two methods, the authors reported that 80.1 % of the positive samples for benzodiazepines by LC-MS/MS were negative (false-negative) by immunoassay. Further, comparison of the methods revealed that 7.5 % of the samples analyzed by the immunoassay resulted in false positives. The study compared the identification of opioids by the two methods, similarly proving a high rate of divergent results.

Due to the advantages of chromatographic methods andtechnological advances, LC-MShas often been reported in the literature as a screening tool for drugs [124,135]. Chromatographic methods are sensitive, specific, featuring a comprehensive approach and using low sample volumes. They frequently do not need the use of analytical reference standards (high resolution mass spectrometry discussed below) and new synthetic drugs may be introduced [52,57,58].

Chromatographic methods have been employed not only for screening but also for drug confirmation and quantification. Papers published between 2015 and 2020 and included in this review (Tables 4 and 5) demonstrated the advancement of methods using high-performance liquid chromatography (HPLC), UHPLC and GC.

Chromatographic techniques have been coupled with Mass Spectrometry (MS) or in tandem Mass Spectrometry (MS/MS). Only the method developed by Jain et al. [136] used a Nitrogen Phosphorus Detector (NPD), whilst Fernández et al. [72] opted for Photodiode Array Detector (PDA).

The chromatographic methods developed in recent years were mostly liquid chromatography, corresponding to 72.7 % of the cases reported in Tables 4 and 5.

Evaluating the methods for determining flunitrazepam, GHB, ketamine, methamphetamine and MDMA, the 2000–2010 survey by Brown and Melton demonstrated that 55.6 % of the reported methods used GC, while 44.4 % used liquid chromatography, if the methods mentioned in the survey are analyzed alone [15]. An increase in demand for liquid chromatography may be observed when the 2010 review is compared with current survey (2015–2020).

The use of gas or liquid chromatography may be justified by the advantages and disadvantages of each technique. GC is widely used in forensic tests due to easy operation, high separation efficiency, selectivity, specificity and sensitivity. Further, it has a lower cost and it is available in most laboratories. However, in sample processing, most of the time, it requires the derivatization process to increase the volatility of analytes. This is a costly process and increases the source of errors [30,48,59,70,74,104,135].

When using liquid chromatography, lower limits of detection and quantification are usually observed due to its high sensitivity rate and specificity. It also detects labile and hydrophilic compounds, presents greater selectivity, and does not generally require the derivatization step. However, in certain cases, the application of liquid chromatography presents restrictions due to a greater consumption of organic solvents, longer analysis time, higher costs, non-availability in some laboratories and difficulty in analyzing volatile compounds [48,52,57,59,65,70,74,86,98,133,137].

When applying the chromatographic method for screening, it is important to note that the CG-MS or CG-MS/MS equipment has a standard library that helps the detection and identification of unknown compounds, while the use of LC-MS requires the feeding of databases and library [21,51,65,138].

Several authors compare the two types of chromatography. Fernández et al. analyzed 17 samples by UHPLC-PDA and GC–MS [72]. The results showed similar or acceptable concentrations by the two methods. In certain cases, the GC–MS method showed greater sensitivity, which may be explained by the detector in liquid chromatography (PDA). However, the derivatization process was indispensable for GC–MS analysis.

La Maida et al., also compared GC and UHPLC, using two types of detectors, MS and HRMS, respectively. UHPLC-HRMS confirmed the presence of original compounds (synthetic cannabinoids) determined by GC–MS and allowed the determination of hydrophilic biotransformation products, undetectable by GC–MS, due to their physical and chemical characteristics [26].

Grapp et al. compared LC and GC using the same type of detector: MS [21]. The authors analyzed 100 serum samples and proved that the LC-MS method was able to detect 240 % more compounds than GC–MS analysis (Fig. 4), attributing this difference to the sensitivity of the instrumentand the drugs'physical-chemical characteristics (very polar,
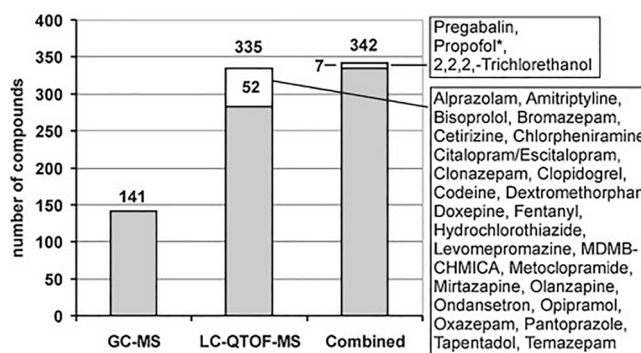

Fig. 4. Number of identified compounds detected by GC–MS, LC-QTOF-MS or a combination of both in 100 authentic serum samples. 52 compounds were exclusively detected by LC-QTOF-MS and 7 compounds could only be detected by GC–MS. Some analytes are cited in the frames. Reference: 21.

non-volatile and thermally unstable substances).

On the oder hand, Cho et al. developed an LC-MS/MS method with an MRM transition mode and obtained a cut-off value for THC-COOH 0.1 pg mg^{-1}, which was greater than that of the GC–MS/MS system (0.05 pg mg^{-1}) [129]. Thus, the authors discuss the recommendation of Kuwayama et al. that LC-MS/MS method could be used to screen for THC-COOH in the hair, prior to confirmatory analysis using GC–MS/MS [129,139].

Regardless of the method chosen, it is extremely important to optimize the technique for the best sensitivity and specificity using the shortest possible analysis time and minimizing the consumption of organic solvents. Important characteristics that have been reported in recent literature for each technique will be discussed separately.

Gas chromatography

The Gas Chromatography (GC) separation technique was most frequently related to Microextraction by a Packed Sorbent, SPME and its variations, DLLME and its variations, LPME and its variations, SPE and LLE (Fig. 2-C).

Derivatization, required by most GC methods, is a process that improves chromatographic behavior and response to drug detection. However, this additional step may be skipped for certain analytes. Several methods for amphetamines, methamphetamines, ketamine, norketamine, cocaine and methadone, for example, skipped the derivatization procedure and still revealed adequate sensitivity in the analysis of biological samples [74,86,87,104].

Since analyteś derivatization may be necessary to increase volatility and make the compounds thermally stable, it becomes relevant when it is intended to analyze molecules with a high molar mass and/or contain strong polar groups. The process consists of a chemical reaction, the most common are alkylation, acylation [98] and silylation [18,59].

Due to its simplicity, versatility and speed, silylation has been the most applied reaction for drug analysis of different types by GC. The reaction takes place in a single step and has high efficiency rates. Different types of acylation agents have been used in recent years, namely *N*-methyl-N- (trimethylsilyl) trifluoro-acetamide (MSTFA) [27,71,127], *N*-methyl-bis-trifluoroacetamide (MBTBA) [128] and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) [88].

For a faster reaction, two other strategies may be employed, or rather, the use of catalysts or the heating of the system. As a catalyst, some methods developed to investigate drugs used trimethylchorosilane (TMCS) associated with the silting agent BSTFA [33,59].

The use of heating to favor effective derivatization has been applied with the use of microwave devices [68,88,127,128]. Prata et al. showed that there is a substantial decrease in analysis time by microwave when compared to conventional processes [127]. The chosen time and power vary according to the protocol. In general, they are used from 2 to 5 min, at 450–800 W.

When derivatization is a necessary process, the method should be optimized to obtain higher performance and increase sensitivity. Among the papers in Table 4, only Orfanidis et al. systematically optimized the method, evaluating the best derivatization time, temperature, and volume of the derivatizing reagent. In the end, the lowest volume of BSTFA with TMCS (50 μL) was used, keeping an intermediate temperature of 70 °C for 20 min [51].

The derivatization stage is considered to be a source of errors because the process is usually manual. Kusano et al. performed derivatization on the chromatographic column itself to minimize the disadvantage [140]. In this procedure, the sample was injected into the instrument and then the derivatization reagent was introduced at a predetermined time. When the vaporized sample entered the column, the target analyte was retained on the column's internal wall. Subsequently, the derivatization reagent flowed through to the column and contacted the adsorbed analyte, consequently derivatizing at an appropriate temperature. The procedure caused a significant improvement in analysis efficiency and performance.

In the development of GC, the choice of the mobile and the stationary phase directly affected the efficiency of the chromatographic separation. The methods developed for drug analysis frequently use helium gas as a mobile phase (Table 4). Helium, compatible with most detectors, is widely used. In fact, it provides better results than nitrogen. However, nitrogen gas is preferred when using flame ionization detectors, like NPD, because it allows lower detection limits. Consequently, the GC-NPD technique, developed by Jain et al. for opiate analysis, had nitrogen as carrier gas [136].

In the case of the stationary phase for determining and quantifying drugs, the long and narrow apolar capillary columns of fused silica [27,33,59,136,140], phenyl-methylpolisiloxane [44,68,71,72,74,86, 87,98,128], diphenyl – dimethylpolysiloxane [104] and silylarylene [65] are commonly used.

The film thickness of the capillary columns directly influences retention time. A lower thickness rate makes the run shorter. Whilst most studies published between 2015 and 2020 included in these review use columns with a 0.25 μm film (Table 4), only one used a thicker column (0.50 μm) [136].

The sample volume injected into the chromatographic system is usually very low, between 1 and 2 μL (Table 4). The GC injector volatilizes the sample and, due to its limitation, small sample volumes are used. If larger volumes are injected, the analytical column may be overloaded or destroyed by the introduction of non-volatile components. The temperature vaporization injector programmed for large volume injection (PTV–LVI) is an alternative to increase sensitivity in the analysis.

The method developed by Ferrari Junior et al. for the quantification of cocaine, MDMA and other drugs, using PTV-LVI, allowed the introduction of 25 μL of the sample. It enabled a sensitivity which may be compared to that by more sophisticated instruments [104].

Liquid chromatography

Most methods that use liquid chromatography apply HPLC (35 %) or UHPLC (24 %) to determine and quantify drugs. The HPLC separation technique was related with higher frequency for LLE, column switching, LPME and its variations, SPE, and DLLME and its variations. UHPC with LLE, SPME and its variations, DPX, MEPS, DLLME and its variations and SPE (Fig. 2-C).

UHPLC emerged with the introduction of columns (stationary phase) containing porous particles ≤ 2 μm in diameter and with smaller dimensions (5 to 10 cm in length; 1 to 2.1 mm for internal diameter). These changes transformed UHPLC into a faster technique, with greater outcome and detectability, besides saving the mobile phase and allowing the introduction of smaller sample volumes. However, the technique requires specific equipment to withstand high pressures (above 1000 bar) [141].

When they developed a UHPLC-MS/MS method for determining Δ^9–THC and its biotransformation products, Saenz et al. reported that the peak profile achieved in the method development was different from that traditionally achieved by HPLC [30]. More acute and narrower peaks were observed, proving the UHPLC's great efficiency.

A current alternative for increasing the sensitivity and robustness of LC methods is the nano flow liquid chromatography (nanoflow-LC) in which mobile phase flows are used in the order of nanoliters/minute, and columns have an internal diameter<100 μm. Limitations associated with the technique have slightly longer runs and additional costs of the pumps [74,128,137,142].

The application of nanoflux-LC for the screening of CBD, cocaine, codeine, MDMA, heroin and other drugs showed an adequate sensitivity of the method for analysis in a urine sample even after the implementation of high dilution factors [143].

Thus, the dimensions of the stationary phase are closely correlated with the efficiency of the chromatographic separation. Moreover, the

type of chromatographic columns is also relevant. The methods developed for drug analysis mostly use C18 as a stationary phase (Table 5). This type of column is classified as chemically bonded, since the octadecyl group is covalently bonded to the silica surface, characterizing it as a nonpolar phase [144].

Another type of chemically bonded column widely used for illicit drug analysis is the columns with the silica-bonded phenyl group (Table 5). Phenyl groups provide a nonpolar feature to the stationary phase [144].

Different stationary phases have different separation mechanisms. Ou et al. evaluated two chromatographic columns: pentafluorfenil (PFP) and C18 with different combinations of the mobile phase [23]. Results indicated that the two types of columns howed huge impacts on peak areas, with the best results being achieved with the PFP column. Another research by Vicenti et al. analyzing the same columns arrived at a similar conclusion: using column C18, some analytes were not completely separated and others were coeluted [49].

In reverse-phase chromatography, performed with nonpolar columns, the mobile phase used must contain more polar solvents (Table 5). Besides being employed for the drugs' physical–chemical characteristics, reverse-phase chromatography eliminates the tail of the chromatographic peaks caused by the adsorption of polar compounds in sites that adsorb the solute [144].

Several authors have also tried to separate isomeric compounds. Christoffersen et al. employed a chiral column to separate the R- and S-enantiomers from methadone [97]. The employed column featured α-1 acid glycoprotein immobilized in spherical silica particles, allowing the separation also in the reverse mode. Maas et al. were able to separate the ortho, meta, and para cathinone isomers using columns with superficially porous particles with a biphenyl group [63].

Several methods have used superficially porous columns to improve the separation efficiency with the use of high flow rates. Superficially porous particles feature a separation comparable to that obtained with totally porous particles without requiring such high pressures [42,63,69].

The normal silica phase has been reported for opioids and cocaine/amphetamine separation [75,145] with researchers employing hydrophilic interaction chromatography (HILIC). HILIC is used to effectively separate small polar compounds using normal phase columns and mobile phases similar to those used by reverse phase chromatography. However, in HILIC, the elution gradient starts with a low polarity organic solvent and elutes polar analytes by increasing the polar water content [18].

Gradient elution is used when a solvent does not provide a sufficiently rapid elution of all its components, requiring the addition of a solvent (aqueous or organic) to increase the strength of the eluent [51]. However, the use of gradient elution promotes pressure oscillations in the chromatographic column and may cause damage. Therefore, when possible, isocratic elution is employed. Only three authors achieved adequate chromatographic separations using isocratic elution within the methods described in Table 5 [75,97,145].

The mobile phase in chromatographic methods for drug analysis consists of an aqueous phase and an organic phase. The aqueous phase is composed mostly of water, while the organic phase is usually acetonitrile and/or methanol. Formic acid may be added to assist analytes ionization when mass spectrometry is used as a detection method (Table 5). Several salts, such as ammonium acetate and ammonium formate, were also added. These salts are considered friendly to the detector, as they are volatile organic salts, easily evaporated or decomposed in the ionization chamber [83].

With regard to the sample volume injected into the chromatographic system, in general, higher volumes than those required for GC analysis were reported. The volumes injected in the liquid chromatography reached 50 µL [135,146]. Several authors performed different injections to evaluate up to 3 different ionization modes (different transitions or ionization in positive and negative mode), also requiring larger sample volumes [42,58,138].

Different analytical methods may be developed for drug screening, where the chosen method is based on the aim of the laboratory analysis [71]. The association of analytical techniques is also possible, as described in a research by Wiergowski et al., in which the general non-targeted screening was performed using HPLC-MS technique and identified substances were quantified by GC–MS and UHPLC-MS/MS technique, according to the physical–chemical characteristics of each analyte [52].

Mass spectrometry

Mass spectrometry is a powerful technique that has been used with chromatography, featuring qualitative and quantitative information, high sensitivity and the capacity of distinguishing between different substances. In the past 5 years, almost all chromatographic methods developed for drug analysis have used mass spectrometry as a detector (Tables 4 and 5).

A mass spectrometer consists of an ion source, which produces ions, a mass analyzer, which separates the ions according to the mass/charge ratio, and an ion detector. New sources of ions and more modern mass analyzers improved the method. When applying the ion source, ion booster (IB), Chepyala et al. obtained an improved response to amphetamines, opioids, cocaine, Lysergic acid diethylamide (LSD) and other drugs in dried blood samples [69].

IB source uses a controlled vaporizer temperature to increase the ionization efficiency of the target analytes, evaporating the solvent, even at high flow rates in the mobile phase. Compared to the traditional source of electrospray ionization (ESI), the main disadvantage of an IB source is that it is not suitable for thermally unstable compounds and the improvement in sensitivity depends on the chemical characteristics of the analytes. In theirstudy, Chepyala et al. compared the two sources and reported that source IB improved the detection sensitivity for 86 % of the analytes by 1.5 to 14 times [69].

Saenz et al. evaluated two different types of ionization sources to verify the response in Δ^9 –THCanalysis and its biotransformation products [30]. Atmospheric Pressure Chemical Ionization (APCI) and ESI were evaluated in positive and negative mode. The ESI source in the positive mode provided maximum ionization for the compounds.

The ESI source is mostly used when coupling MS to liquid chromatography. However, the effects of the matrix are evident in such ionization. Consequently, so that interference could be minimized, lower flows, such as nanospray, were suggested, which provided a significant increase in ionization efficiency when compared to pneumatically assisted sources. Further, the use of nanospray also offers advantages in terms of tolerance to signal suppression.

Due to increased sensitivity, two methods were developed for drug analysis using nanospray and achievedsuitable results for the analysis of diluted biological samples [137,143].

In the case of mass analyzers, high resolution mass spectrometry (HRMS), using Time-of-Flight analyzers (TOF) and Orbitrap, has experienced in recent years a significant growth in many laboratories due to their high selectivity and robustness. They may also provide precise masses that determine the formulation of elementary compositions. The combination of LC or GC and Orbitrap provides a quick resolution of this matrix of compounds in a relatively short run time [57].

HRMS applications in drug analysis have shown that isobasic interference has decreased significantly. Increase in mass accuracy allowed the identification of substances and reduced the number of chemical formulas. Further, the technique provided excellent sensitivity, even if the analyses were carried out in the scan mode and the fast switching from positive/negative ionizationwas also possible, expanding the range of analyzed substances. Thus, the hyphenation of the chromatography with HRMS showed great potential in wide-spectrum screening procedures from low volumes of biological matrices. However, the disadvantages of Orbitrap are still high cost and complex operation

[48,62,65].

Another strategy to improve sensitivity has been the hyphenation of more than one analyzer, generating a hybrid equipment, such as the Quadrupolar-Time of Flight analyzer (QTOF). The use of QTOF-MS with independent data acquisition provides a powerful tool for comprehensive drug testing and it is also an effective method for detection and confirmation [21,69].

QTOF-MS was used by Palmiquist et al. for opioid analysis. The authors also evaluated two analyzers: TOF and All Ions Fragmentation (AIF) [138]. The TOF mode proved to have lower limits of detection for all analytes based on the detection of a precursor ion. However, the AIF mode may help in the analysis of a true unknown, obtaining a collaborative spectrum with product ions produced at various collision energies.

By using QTOF-MS, Ibanez et al. analyzed biotransformation products for which there were no available standards [146]. The approach supposed a common fragmentation pathway for the original metabolites and the illicit drugs. The authors also needed to use tandem mass spectrometry with the generation of several fragments (MS^n) to induce low and high energy fragmentation. MS^n experiments were greatly important to guarantee the success of the analysis since it allowed the fragmentation of compounds in the collision cell without the previous selection of precursor ions.

Due to excellent selectivity, better specificity and limits of detection, MS/MS has been widely used in drug analysis. MS/MS has been coupled to GC and HPLC/UHPLC, with adequate analysis for several drugs (Tables 4 and 5) [57,140].

In the case of the ionization mode, the full scan mode is normally used only in the selection of ions during the development of the method or in the screening methods. The full scan allows a wide range of masses to be scanned, generating a co-elution of interfering compounds that impair the analysis of specific compounds. Sensitivity is affected when this ionization mode is employed [147,148].

When MS is used, the quantification of the analyte is carried outin the Selected Ion Monitoring (SIM) mode, which allows the selection of a small range of masses (m/z) ormass unit rate. There is an increase in selectivity in the SIM mode, with improvement of sensitivity and decrease in noise. Most of the GC–MS methods in recent years that have analyzed drugs have opted for ionization using the SIM mode [27,33,44,59,68,71,75,86,104,128].

MS/MS methods prioritize the mode of ionization by Multiple Reaction Monitoring (MRM) [31,32,48,66,88] or Selected Reaction Monitoring (SRM) [58,83,98,133]. In these ionization modes, the precursor > fragment pair is monitored, making it more selective than the SIM mode. SRM mode obtains data only for one or more specific product ions produced. On the other hand, MRM mode applies selected reaction monitoring to multiple product ions from one or more precursor ions [88].

Meng et al. evaluated the MRM and SIM mode for GHB analysis [88]. As expected, the SIM mode showed interferences caused by the impurities of the derivatization reagents whilst sensitivity decreased significantly. MRM mode was chosen to improve detection sensitivity.

Sometimes, two differentes modes of ionization could be apllied, like the method developed by Cho et al. The authors used a LC-MS/MS system with two different acquisition modes (MRM and MS^3) to identify and quantify THC-COOH in the hair. In this case, MRM was first used for identification and quantitative analysis, while MS^3 was used for reconfirmation detection. The association of the two modes was useful to increase the reliability [129].

So that sensitivity would be improved, Klima et al., analyzing morphine, codeine, cocaine, MDMA, amphetamines and other drugs, performed a post-column addition of 2-propanol at a 0.1 mL min^{-1} flow rate, favoring the ionization of the analytes [31].

Novel approaches in analytical techniques

The sensitivity and specificity of some mass spectrometry techniques have allowed the isolated application of the method without the previous need for chromatography.

Direct analysis by real-time mass spectrometry (DART-MS) is a powerful tool, and although commonly considered a screening technique, it has advanced along with sample preparation techniques, focusing mainly on SPE and its variations, as SPME, showing itself capable of providing quantitative or confirmatory results [149].

In recent years, mainly due to the development of ion sources of environmental ionization and direct sampling methods, mass spectrometry has been used for rapid methods of drug screening [134,150–153].

One method used a miniaturized portable mass spectrometer with a Linear Ion Trap (LIT) and, for sampling, a paper-capillary spray cartridge. By applying the technique, the authors were able to properly screen drugs from urine samples in a process that took about 2 min and could be performed on the spot [134].

The method developed by McKenna et al. used the paper spray to prepare the sample with HRMS analysis [150]. The semi-quantitative method analyzed 137 drugs and their biotransformation products in blood samples in just 2.5 min. Other authors have also used paper spray mass spectrometry (PS-MS) after slug-flow microextraction (SFME) for rapid analysis of drugs of abuse (AMP, MA, MDMA) in whole blood and urine samples [152].

Ng et al. used wooden-tip electrospray ionization mass spectrometry (WT-ESI-MS) for the detection and quantification of drugs of abuse in urine and oral fluid, in an approximate time of 4 min [151]. Validation was satisfactory for all drugs analyzed, except for THC, requiring optimization of the analytical method.

Although the DART-MS technique is most frequently applied to urine, oral fluid and whole blood samples, the biggest question is its functioning in cases of authentic samples [149]. It is a versatile technique that operates in open air, allowing for rapid, noncontact analysis of solid, liquid, and gaseous materials without any pretreatment of samples, it has high yield and lack of memory effect [153]. However, partnerships between chemical researchers and forensic toxicologists are needed to understand their main limitations [149].

Conclusion and future challenges

Identification and quantification of drugs of abuse in biological matrices plays a very important role in clinical and forensic cases. Health and social problems, injuries caused by violence and road accidents due to driving under the influence of drug, unprotected sex, facilitated crimes and deaths due to drug addiction are worth mentioning. Consequently, information on the advantages and advances with regard to the choice of biological samples and the most efficient analytical method is necessary.

In order to choose the best biological sample, the most adequate sample preparation method and the ideal chromatographic conditions, the analyst must take into account several factors, such as: (1) purpose of the analysis (is there an urgent need to obtain results? is it necessary to maintain strict chain of custody for forensic application?); (2) characteristics of the analytes to be studied (are the analytes volatile? Are the drugs to be analyzed polar or apalores?); (3) availability and viability of the sample (which samples are available? Do the toxicokinetic characteristics allow for the detection of the analyte in this biological sample? How will transport be carried out? Does the laboratory have equipment with sufficient capacity to find the concentration of the analyte in this sample?); (4) analytical techniques (is the sample preparation method applicable in the laboratory routine? Is the analysis time long? Is a specialized technician necessary to conduct the analysis? Does the laboratory have the necessary basic equipment? Is there a reduction in environmental impacts?). Thus, defining the best analytical method for

identifying and/or quantifying drugs of abuse to be applied in the local reality will include considering the points listed above and correlating them with the advantages and disadvantages of each technique/ sample.

The large number of NPS, added to polydrug consumption in the "chemsex" context, which results in different classes of drugs that may have related chemical structures, is a constant challenge for analysts. This scenario reinforces the main focus of this review, bringing the reader current trends in biological sample preparation, drug identification and quantification, especially in alternative specimens. Thus, with this review, we identified a growing development of multidrug techniques, that is, the literature has prioritized the analysis of several drugs of abuse and their biotransformation products in the same analytical method. It was evident that the choice of technique for sample preparation or the definition of the detection method does not have a direct correlation with the physical and chemical characteristics of the analytes. However, these characteristics directly influence the optimization of techniques, such as pH definition, sorptive phases, mobile phases, among others.

Regarding biological samples, whole blood and urine are still widely used in these research works. Future perspectives concerning the increase of dry samples analysis, not only in forensic analysis but also in other areas, such as clinical application, may be surmised. This perspective is based on the advantages of these samples and the possibility of requesting a smaller amount of biological sample. In particular, the use of these matrices will enable them to apply direct methods, such as the use of paper spray mass spectrometry.

In the preparation of samples, Toxicological Analysis has tried to be more refined, with increasing improvements in availability and sensitivity to toxicological data. In summary, there are several methodologies, some more current, that arise from innovative technologies and may provide greater environmental and economic advantages. In the last five years, there has been an increase in the search for miniaturized techniques, either LLE or SPE.

Most of the methodologies for sample preparation applied in the last few years showed an outstanding concern with green chemistry, that is, they aimed at the technique's miniaturization. However, another innovation that deserves to be widely explored in the future refers to the automation of methods. The application of online techniques was reported in a few articles used in current review. Automation could generate less human handling of the samples and, consequently, less chance of errors. Moreover, automation makes analysis faster and requires shorter time by the forensic analyst.

It is worth mentioning that when choosing an analysis, as discussed previsly in this narrative review, special attention should be given to the limitations imposed, challenges, type of biological matrix and availability. Further, when defining a methodology for sample preparation, the sensitivity required for later detection must be achieved. Thus, it must be a recurring concern for the toxicologist to expand the limits of detection and quantification of different drug groups, especially with regard to the elaboration of evidence in criminal cases, such as drug-facilitated crimes.

The new techniques for preparing biological sample are important tools for the pre-concentration of analytes. However, it is necessary to use sensitive detection methods with good resolution and peak capacity. Although still in a solid position due to the wide availability in forensic analysis laboratories, GC has been replaced in the last five years by liquid chromatography (UHPLC and HPLC). Some LC methods do not require lengthy or complicated sample preparation or derivatization and are therefore widely used. In the case of the detector, there is an increasing search for the use of HRMS instruments due to the high selectivity and robustness. Additionally, it provides precise masses which determine the formulation of elementary compositions. Consequently, it is an important ally in the challenging analysis of new synthetic drugs. Mass spectrometric analysis (MS^n), in several stages, has also been important in these cases. Therefore, innovations in strategies related to mass spectrometry in drug analysis are believed to be quite promising. It is also worth mentioning that new technologies to expand portable MS equipment are expected to allow the analysis of multiple drugs *in loco*.

The availability of increasingly sensitive analytical methodologies is expected. Laboratories are always challenged to detect and quantify new drugs, in addition to those already known. Thus, the choice of the best sample preparation method, the detection and quantification technique, and conventional or alternative specimens are crucial. The analyst must always have in mind the need to optimize the variables involved in all stages and start to expand the use of chemometric tools. Further, before being applied for the preparation of forensic reports, analytical validation of the method based on national and international guidelines is essential.

Compliance with ethical standards.

Conflict of interest. The authors declare that no confict of interest exists.

Human and animal rights. This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2022.100442.

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