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Recent chromatographic and electrophoretic based methods for determining drugs of abuse in urine and oral fluid: A review from 2018 to June 2021

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ABSTRACT

Urine and oral fluid are important biological matrices used for forensic and toxicological analyses. These two complementary matrices can be used to provide information about recent and long-term drug consumption. Several analytical methods have been developed in recent years to determine different drugs of abuse in these biological samples. Most of them are based on chromatographic and related techniques, such as liquid chromatography or gas chromatography and capillary electrophoresis. Moreover, as these biological matrices can contain various compounds that may interfere with the analytes of interest, a sample pre-treatment is usually necessary. Different pre-treatment strategies have been carried out, including solid-phase extraction and liquid-liquid extraction, among others. The present review aims to provide a comprehensive overview and a discussion of the latest trends and the most used strategies for determining drugs of abuse in urine and oral fluid samples using chromatographic and electrophoretic techniques between 2018 and June 2021.

1. Introduction

Drugs of abuse (DOAs) have been a matter of concern for a long time due to their increasing use and negative impact on society. During the last decade, there has been an expansion in the drug market, mainly due to the constant introduction of new synthetic drugs, such as new psychoactive substances (NPS). Some of these synthetic substances mimic the effects of traditional drugs, and include stimulant, psychedelic, hallucinogen and depressant substances, and can be easily found on different websites [1–4]. DOAs can be classified in different ways but the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) classifies them in their annual report as cannabis, cocaine (COC), amphetamine-type substances (ATS), NPS, heroin (HER) and other opioids, and finally, other drugs. For example, in the case of cannabis, compounds such as Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) can be found. In the case of ATS, the most known substances are amphetamine (AMP), methamphetamine (MAMP) and 3,4-methylenedioxymethamphetamine (MDMA or ecstasy). Morphine (MOR), codeine (COD) and methadone (MTD) are some of the most known opioids on the market. In the NPS group, synthetic cathinones, synthetic cannabinoids and benzodiazepines (BZD) are the most consumed substances. Other DOAs that are also popular are ketamine (KET) and lysergic acid diethylamide (LSD), among others [5,6]. Moreover, it is important to highlight that some of these compounds may have a chiral centre, and thus, two enantiomeric forms. As each enantiomer can have different pharmacokinetic and neuro-pharmacological properties, their behaviour in the human body can be completely different [7,8]. For example, in the case of AMP, the S enantiomer shows therapeutic properties to treat attention-deficit hyperactivity disorders (ADHD), while the R enantiomer is responsible for the psychoactive effects [9].

DOAs are continuously monitored and every year some of the new drugs that have appeared are included in the list to be controlled by the EMCDDA in Europe or by the United Nations Office on Drugs and Crime (UNODC). These two organisations implement Early Warning Systems (EWS) to detect DOAs, and more precisely, NPS, which are the new substances constantly appearing on the market. Moreover, these organisations cooperate with other regions, such as Latin America and Asia, to prevent the manufacture of drugs and their entrance into America or Europe [5,10].

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In 2017, and due to the large increase in these drugs, new laws were adopted to regulate them better [6,11]. This new legislation focused on reducing deadlines and simplifying the administrative procedures to minimize the time for controlling a substance. This was an important advance compared to the previous actions, which involved laborious procedures, so that by the time one substance was controlled, a different substance had already been introduced onto the market. Although this is still happening, the time needed to start controlling a drug has been considerably reduced.

Determining these drugs in biological samples is very challenging due to the growing number of compounds with new chemical structures that can be found on the drug market and also because of the complexity of biological specimens. Once the drugs are ingested, they rapidly reach all parts of the body through the blood. Then, they are metabolised in the liver because they are lipid soluble, and finally, they are eliminated from the body through the urine [12]. The most common biological specimens for toxicological and forensic analyses are blood (including plasma and serum), urine, oral fluid (OF) and hair. The choice of the biological sample is crucial because it plays an important role in the obtained results. Each matrix reflects a different temporal window, which can be related to the time of drug intake. For recent consumption, matrices such as blood or OF are generally used. For mid-term consumption, urine is the most employed specimen since it can cover from minutes to weeks. Finally, to detect a drug consumed months ago, hair is the best matrix, and it can also be useful to monitor a person consumption along time, because hair grows approximately 1 cm per month and the analysis of different hair segments can give information about the last use. Other differences to be considered among biological samples are related to the invasiveness of the collection, the costs and difficulties of the analysis, the reproducibility of the results and the possibility of interferences. In the literature related to these topics, blood and urine have been historically the most analysed biological matrices to detect use or abuse of drugs, but in the last years, other specimens can help toxicologist to answer important questions related to drug consumption. It is the case of, which can be used as an alternative to blood, displaying a narrow window of a few hours [12–16]. Considering that urine is the most common matrix for drug testing, offering a mid-term window of detection and that OF has become an excellent alternative to blood for recent consumption, the present review is focused on these two matrices. Their main advantages compared to other widely used matrices, such as blood, is that they are very easy to collect and specialized personal are not required. Sometimes, when DOAs are determined in biological samples, the parent drug is not always found and therefore it is important to determine their metabolites. In general, by determining both the parent and the metabolites, recent drug use or a past consumption can be differentiated because in the first case the concentration of the metabolite is very low or it is not found, while in a past consumption the parent drug is not detected or it is detected at a very low concentration; however, this depends on the drug metabolism and on different parameters, such as the concentration taken [12,15–17]. Some of the metabolites of the most consumed DOAs are benzoylecgonine (BZE) for COC, 3,4-methylenedioxyamphetamine (MDA) for MDMA, AMP for MAMP, 11-hydroxy-THC (THC-COOH) for THC and 6-acetylmorphine (6-AM) for MOR, among others.

The EMCDDA, UNODC and the authorities of each country usually control the DOAs with screening tests [1,12,18]. These tests are based on an initial qualitative test followed by a confirmation test, which is performed to avoid false positives. Initial tests are qualitative analyses that indicate the presence or absence of a certain substance. They use easy equipment based on immunoassay techniques with rapid results and they do not need trained technicians, unlike confirmatory tests. The confirmatory tests provide quantitative results with high specificity and sensitivity. They are based on chromatographic and related techniques mostly coupled to mass spectrometry (MS) [12]. Different research groups have focused on determining DOAs in biological samples using the mentioned instrumentation. The most used techniques are gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) [15–17,19]. The general trend is to combine these techniques with MS, tandem MS (MS/MS) or high-resolution MS (HRMS) to improve the sensitivity of the method to achieve the levels at which these drugs are usually present in biological samples (ng mL^{-1}).

The main objective of the present review is to critically discuss the chromatographic based and related methodologies developed to determine the most usual DOAs in urine and OF between 2018 and June 2021. In the last years, different reviews have been published related with to this topic, however, they are mainly focused in a specific family of DOAs, or also there are some examples focused in a particular DOA, matrix or instrumentation technique [20–26]. For example, Amini et al. [20] or da Silva et al. [22] focused their reviews on different strategies devoted to the determination of MOR and its metabolites, or ecstasy, respectively. Baciu et al. [24] focused their publication on a particular matrix, hair, highlighting the pros and cons of different strategies for the determination of different DOAs using separation techniques. In our case, the main objective of this review is to provide an update on the pre-treatment procedures, chromatographic and electrophoretic techniques and main findings in the analysis of DOAs in the two selected biological matrices. Even that, as it has been mentioned, there are some interesting reviews focused on related topics, a comprehensive review including all types of DOAs, different pre-treatments, analysis techniques and matrices is needed. Moreover, an important purpose it is also to provide insights for the future research directions of analytical methods related to DOAs determination. The review is divided into two main sections, one related to urine and the other in OF. Each section is also divided into two subsections: the first discusses the main pre-treatment procedures while the second part focuses on the use of separation techniques such as LC, GC and CE and the compounds found in biological specimens from drug users.

2. Urine

Urine is one of the most commonly used biological matrices for toxicological drug analysis and also one of the most used in the forensic field [12,15,16]. Once the drug is taken, it is absorbed in the tissues and excreted through the urine. In this matrix, the metabolites, and to a lesser extent the parent drug, can be found during the days after drug consumption. This is because urine has a wide detection window that can vary from minutes to weeks, even that this can depend on the individual metabolism and the type of consumption. For example, chronic exposures can be detected after one week of the intake [12]. Therefore, urine can be used to determine both recent and mid-term consumption. More advantages of urine are that large amounts of sample can be collected, specialized personal are not necessary, sampling is non-invasive and easy and rapid responses can be obtained with immunoassay tests. They are inexpensive, easily automated and can be focused on detecting drug families or particular DOAs. These immunoassay tests provide qualitative and presumptive results and, in case of positive results, these must be confirmed through more sophisticated analytical methods. However, urine has also some drawbacks, being its main inconvenient that it is easy to adulterate because of the non-observable collection, and if collection has to be supervised, then it is invasive for the donor [12,15,27,28]. The

methodologies used for determining DOAs in urine usually involve three main steps: sampling, pre-treatment and analysis. Table 1 shows the main details of the most recent methods for determining DOAs in urine by chromatographic and related techniques.

2.1. Urine sample pre-treatment

Urine is usually collected in a clean, sterile container and as a first step in methods for determining DOAs, some authors centrifugate it to eliminate possible insoluble materials. Then the urine can be frozen at −20 °C in small aliquots to avoid freezing-and-thawing [29]. Some authors perform a protein precipitation, and the most employed reagent is trichloroacetic acid (TCA) [29,30]. Other authors use the dilute and shoot strategy, in which urine is diluted directly with an aqueous solution and then injected directly for analysis [31–34]. For example, Kavanagh et al. [31] and Olesti et al. [32] diluted the urine with an aqueous solution with ACN and MeOH, respectively, prior to the sample analysis. There are other examples in which another step is carried out before this dilute and shoot strategy, as for example Fan et al. [33] who centrifuged the urine first to collect the supernatant and then diluted it with a 50% MeOH aqueous solution before analysing it. However, it is important to mention that these strategies are useful for identifying these compounds or for quantifying them at high concentration levels. Moreover, even that the possible interferences present in the matrix are reduced with this kind of strategies, they are not completely removed and the remaining interferences can interfere with the chromatography and ionisation of the target analytes. However, a challenging item is to establish the proper dilution factor, since this is dictated by many factors including the expected concentration of the analytes, the matrix analysed or the required selectivity and sensitivity [35].

In order to eliminate these possible interferences, a more exhaustive pre-treatment step is usually required. In this regard, hydrolysis can be applied in some cases as a single pre-treatment technique to liberate free drugs from their metabolised conjugates. In recent years, this has been used to split the possible glucuronide conjugates present in the urine and it has mainly been performed with β-glucuronidase enzyme or with sodium hydroxide (NaOH) [34,36–41]. For example, Kahl et al. [34] used β-glucuronidase to perform an enzymatic hydrolysis before the dilute and shoot strategy without any interference to their analysis. For example, β-glucuronidase is very useful for some opiates as MOR, as most of the administered MOR (87%) is eliminated through urine in 72 h and 75% of this is excreted as MOR 3-glucuronide [42]. In other reported strategies, other alternatives have been employed after the hydrolysis process, such as diluting or extracting the analyte solution using LLE or SPE to remove the interferences and preconcentrate the analytes to achieve lower concentration levels. Anzillotti et al. [39], in an acute toxicity study, developed a method for determining mephedrone based on hydrolysis with β-glucuronidase, incubation and then a LLE step. They concluded that there is a lack of information about the acute and chronic toxicity of this substance. Al-Asmari et al. [37] also used hydrolysis, but with NaOH, and then they performed a SPE process for the extraction of THC and its metabolites, achieving recoveries from 83 to 97%. However, the main drawback of these developed strategies is that they combine various sample pre-treatments and even that cleaner extracts are achieved, the analysis time is also considerably increased.

LLE is one of the most employed sample pre-treatment techniques and has also been used as a single sample pre-treatment by some authors [39–41,43–52]. Its main advantages are its simplicity and the wide availability of organic solvents. The most used organic solvent for DOAs extraction in recent years has been ethyl acetate [39,45,46] or a combination of ethyl acetate and other organic solvents, such as hexane or 2-propanol (IPA) [40,48,49]. This is the case of Kim et al. [45], who performed a LLE using ethyl acetate as organic solvent, and achieved recovery values between 69 and 96% for 13 amphetamine-related NPS. Moreover, some authors have added a salt to salting-out LLE (SALLE), and in these cases NaCl is the most used [47,50]. For example, Pérez-Alcaraz et al. [50] evaluated adding NaCl to the LLE of three synthetic cathinones from urine using toluene as organic solvent. Better recoveries were obtained when NaCl was added to toluene (between 33 and 65%) compared to that obtained without the salt (between 21 and 51%). Although LLE is a widely employed technique due to its simplicity, the trend nowadays is to reduce the volumes of organic solvents generally employed to obtain more environmentally friendly methods. In this regard, some authors have employed microextraction techniques, such as dispersive liquid-liquid microextraction (DLLME) [41,53,54]. For example, Mercieca et al. [41] developed a method for determining ATS and cathinones by DLLME followed by GC-MS, achieving high recovery values (between 92 and 115%) but using chloroform as an extractant solvent. The developed miniaturised strategy achieved excellent results, reduced the analysis time and even that the application of this kind of microextraction techniques is still limited to research, their use is gaining more attention in forensic laboratories, specially DLLME since it can be easily performed without needing the purchase of special devices.

SPE is the most used pre-treatment technique in recent years for DOAs determination in urine [37,44,49,55–66]. In this case, a wide variety of sorbents have been used, which include commercial sorbents and also homemade sorbents. In the reported literature, although some authors use silica-based sorbents, polymeric sorbents are the most employed. Within these, mixed-mode sorbents are usually the most used because in general, most DOAs are basic compounds and at a pH lower than their pK_a, they are charged. For example, Pascual-Caro et al. [55] compared a weak (Oasis WCX) and a strong (Oasis MCX) mixed-mode cationic-exchange sorbent for determining a group of synthetic cathinones in urine. In both cases, the extraction efficiencies were higher than 84% for all the included cathinones. However, the matrix effect was considerably lower (between 6 and 29% lower) for the strong mixed-mode sorbent, so the authors selected this one. Musile et al. [60] synthetized an in-house mixed-mode cation-exchange sorbent for determining 16 DOAs, including ATS, COC and opiates. The obtained recoveries were compared with those obtained with the two mixed-mode cation-exchange commercial sorbents. The authors concluded that better extraction efficiencies were obtained with the synthetized sorbent. Values of between 50 and 114%, were achieved showing high versatility for the compounds studied. Some authors use SPE with highly selective sorbents. This is the case of Al-Asmari et al. [37], who used a Clean Screen THC sorbent, which is a mixed-mode anion-exchange sorbent specially manufactured for extracting THC and its metabolites. They achieved excellent extraction efficiencies with recoveries from 83 to 97%, demonstrating the high selectivity of the sorbent used.

Some authors have also used reversed-phase sorbents, although they are not as used as the mixed-mode sorbents. For example Gaunitz et al. [56] used reversed-phase sorbents in the method they developed for determining 61 synthetic cannabinoid metabolites by a SPE using Strata Phenyl cartridges with recoveries from 43 to 97%. Another example is Cheng et al. [65], who used Oasis HLB cartridges for the SPE extraction of α-PVP and its metabolite with recovery values of 88 and 99%, respectively. Generally, the washing step in these cartridges is not as exhaustive as in the case of mixed-mode sorbents due to the pH strength. For example, Gaunitz et al. [56] used a mixture of MeOH:H_2O (5:95, v/v), while for the mixed-

Table 1
Analytical methodologies for the determination of drugs of abuse in urine samples.

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
α-PVP and OH- α-PVP	GC-MS	Extraction: SPE (Oasis HLB) Derivatisation: BSTFA and pyridine	88–99%	LOD: 5 and 25 ng mL^{-1} LOQ: 25 ng mL^{-1}	1 positive in α-PVP (67.3 ng mL^{-1}) and OH-α-PVP (560.2 ng mL^{-1})	[65]
CBN CBD THC	GC-MS	Hydrolysis: 1 M NaOH Extraction: LLE (ethyl acetate/hexane)	91–117%	LOD: 1.0 ng mL^{-1} LOQ: 2.5 ng mL^{-1}	Analysis of hemp seed of Korea. They do not cause positive in urine tests	[40]
8 ATS and synthetic cathinones	GC-MS	Extraction: SPME Derivatisation: PFPA Incubation: 60 °C 10–15 min	2–80%	LOD: 5–25 ng mL^{-1} LOQ: 25–100 ng mL^{-1}	3 positive cathinones (227–1209 ng mL^{-1})	[127]
ATS Cathinones Phenethylamines Ketamine analogues	GC-MS	Hydrolysis: 0.2 M NaOH Derivatisation: hexyl chloroformate Extraction: DLLME (chloroform/MeOH)	92–115%	LOD: 1–50 ng mL^{-1} LOQ: 2–50 ng mL^{-1}	Rapid and cheap determination with flash derivatisation. Only 6 min of total sample processing	[41]
Tramadol and MTD	GC-MS	Dilution: basic buffer Extraction: synthetized layered double hydroxide	88–92%	LOD: 0.15–0.45 ng mL^{-1}	Easy magnetic separation and large adsorption capacity with the nanocomposite adsorbent	[74]
29 cathinones and ATS	GC-MS	Extraction: SPE (ZSDAU20 Clean Screen) Derivatisation: PFPA	80–120%	LOD: 0.5–10 ng mL^{-1} LOQ: 5–50 ng mL^{-1}	Valid as a confirmation test for 20 out of 29 drugs	[73]
Acetyl fentanyl	GC-MS	Extraction: SPE (ware Cerex Trace-B)	–	LOD: 0.75 ng mL^{-1} LOQ: 2.5 ng mL^{-1}	19 positive samples in acetyl fentanyl (41–9825 ng mL^{-1})	[63]
18 cathinones and 4-fluoroamphetamine	GC-MS	Extraction: LLE (tert-butylmethylether) Derivatisation: TFAA	–	LOD: 10–30 ng mL^{-1} LOQ: 100 ng mL^{-1}	594 samples: 7 positive: 4 in butylone (0.8–8.4 mg L^{-1}), 1 in 4-FA (0.1 mg L^{-1}), 1 in mephedrone (81 mg L^{-1}) and 1 in 3-MMC (43 mg L^{-1})	[43]
AMP MAMP MDA MDMA MBDB MDE	GC-MS	Extraction: MEPS C_{18} Microwave derivatisation: MBTFA	19–71%	LOQ: 25–50 ng mL-1	First study to determine ATS in urine by MEPS followed by GC-MS	[67]
23 NPS including cathinones	GC-MS	Extraction: LLE (phosphate buffer) and SPE (Clean Screen ZSDAU020) Derivatisation: PFPA	64–105%	LOD: 0.2–1.0 ng mL^{-1} LOQ: 0.5–20 ng mL^{-1}	Better application in blood than in urine	[44]
13 AMP-related NPS	GC-MS	Extraction: LLE (ethyl acetate) Derivatisation: TFAA	69–96%	LOD: 0.4–2.5 ng mL^{-1} LOQ: 2.0–25 ng mL^{-1}	7 samples: AMP (39–499 ng mL^{-1}), MAMP (106–621 ng mL^{-1}), MDMA (445 ng mL^{-1}) and MDA (17 ng mL^{-1})	[45]
Mepirapim Acetyl fentanyl	GC-MS, GC-QqQ and LC-QqQ	Extraction: SALLE (diethyl ether)	87–89%	LOD: 0.1–1.0 ng g^{-1} LOQ: 10–20 ng g^{-1}	First study of mepirapim in blood and urine matrices	[47]
Mephedrone	GC-MS and LC-MS/MS	Hydrolysis: β-glucuronidase Incubation: 45 °C overnight Extraction: LLE (ethyl acetate)	–	LOD: 10 ng mL^{-1} LOQ: 20 ng mL^{-1}	1 positive of mephedrone (2 mg L^{-1})	[39]
18 BZD	GC-MS and LC-QTrap	Extraction: Salle-hybrid PPT/SPE	42–118%	LOD: 2.5–12.5 ng mL^{-1}	185 samples: zolpidem (49), acetaminophen (29) and citalopram (16)	[64]
AMP MAMP Phentermine MDA MDMA MDEA	GC-QqQ	Extraction: LLE (ethyl acetate) Derivatisation: TFAA	91–104%	LOD: 0.09–0.45 ng mL^{-1} LOQ: 0.26–1.40 ng mL^{-1}	22 samples: AMP (0.6–169 μg mL^{-1}), MDMA (0.14–37.7 μg mL^{-1}), MDA (0.37 μg mL^{-1}) and MAMP (0.9 μg mL^{-1})	[46]
14 synthetic cathinones	GC-QqQ	Extraction: SPE (CSDAU203 Clean Screen) Derivatisation: L-TPC	88–100%	LOD: 0.26–0.76 ng mL^{-1} LOQ: 0.86–2.51 ng mL^{-1}	Enantiomeric separation of 14 synthetic cathinones in 60 min	[75]
5 antipsychotics	LC-QqQ	Extraction: HF-based-SPME	65–102%	LOD: 6.25–12.5 pg mL^{-1} LOQ: 12.5–25 pg mL^{-1}	Study of four types of HF membrane materials	[76]
61 cannabinoids	LC-QqQ	Protein precipitation: ACN Extraction: SPE (Strata Phenyl)	43–97%	LOD: 0.025–0.5 ng mL-1	61 samples: 1 positive in ADB-PINACA N-pentanoic acid	[56]
52 DOAs including ATS, opiates, COC, BZD and THC	LC-QqQ	Hydrolysis: β-glucuronidase Dilution: mobile phase	–	LOD: 2–20 ng mL^{-1}	Comparison of the developed method with the stablished ELISA immunoassay methodology	[34]
73 synthetic cathinones and metabolites	LC-QqQ	Urine centrifugation Dilution: MeOH:H_2O (50:50, *v/v*)	–	LOD: 0.1–0.5 ng mL^{-1} LOQ: 0.5–1.0 ng mL^{-1}	67 samples (32 positive). Mephedrone was the most detected analyte	[33]
11 synthetic cathinones	LC-QqQ	Extraction: SPE (AFFINILUTE MIP-AMP)	61–91%	LOD: 0.01–0.13 ng mL^{-1}	Application of an ATS MIP to synthetic cathinones	[77]
14 NPS	LC-QqQ	Dilution: MeOH:H_2O (15:85, *v/v*)	85–115%	LOD: 0.3–2.5 ng mL^{-1} LOQ: 1–5 ng mL^{-1}	Simple dilute and shoot strategy	[32]
44 synthetic cannabinoids	LC-QqQ	Extraction: LLE (K_2CO_3 and 1-chlorobutane)	21–99%	LOD: 0.01–4 ng mL^{-1} LOQ: 0.02–10 ng mL^{-1}	500 samples: 108 positives: 5F-NPB-22 (4.58–137.33 ng mL^{-1}) and S-AB-FUBINACA (0.06–76.50 ng mL^{-1})	[78]

Table 1 (*continued*)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
50 NPS	LC-QqQ	Extraction: LLE (NH_4OH)	–	LOD: 2.5 ng mL^{-1}	110 samples (28% positive and 60% in traditional drugs)	[79]
COC and opioids	LC-QqQ	Method 1: Dilution and incubation. Centrifugation and SPE (ZSDAU200 Clean Screen) Method 2: Protein precipitation. Centrifugation and freezing	(1) 66–93% (2) 37–71%	LOD (1): 0.12–5.0 ng mL^{-1} LOQ (1): 1–10 ng mL^{-1} LOD (2): 0.06–2.5 ng mL^{-1} LOQ (2): 1–10 ng mL^{-1}	Comparison of both extraction methods. Method 2 was the method used but method 1 was implemented due to the good results	[80]
THC THC-COOH THC-OH	LC-QqQ	Hydrolysis: 10 M NaOH Extraction: SPE (Clean Screen THC)	83–97%	LOD: 0.5–1.3 ng mL^{-1} LOQ: 1–2 ng mL^{-1}	25 samples: 25 positives in THC-COOH (224 ng mL^{-1}), 4 in THC (4 ng mL^{-1}) and 7 in THC-OH (4 ng mL^{-1})	[37]
Desomorphine	LC-QqQ	Extraction: SPE (PolyChrom Clinn II)	90%	LOD: 0.5 ng mL^{-1} LOQ: 0.5 ng mL^{-1}	Isolation of desmorphine with high recoveries	[59]
Cannabinoids	LC-QqQ	Extraction: MIP μ-SPE	86–106%	LOD: 0.032–0.748 ng mL^{-1} LOQ: 0.107–2.5 ng mL^{-1}	Fast pre-treatment due to the simultaneous use	[61]
10 cathinones	LC-QqQ	Extraction: MIP-μ-SPE US (heptane/IPA/ammonium hydroxide)	87–102%	LOD: 0.14–1.51 ng mL^{-1} LOQ: 0.48–5.03 ng mL^{-1}	MIP synthetized with high selectivity for cathinones	[62]
BZD	LC-QqQ	Hydrolysis: β-glucuronidase Protein precipitation: cold MeOH	56–114%	LOD: 2.5–5 ng mL^{-1} LOQ: 5–100 ng mL^{-1}	60 opioid-positive samples finding BZD in 8 of them. Evaluation of three protein precipitation plates	[38]
77 NPS, 24 classic drugs and 18 metabolites	LC-QqQ	Dilution: M3® buffer	>80%	LOD: 0.02–0.25 ng mL^{-1} LOQ: 0.06–0.5 ng mL^{-1}	23 samples: MOR (180 ng mL^{-1}), COD (85 ng mL^{-1}), 6-AM (67 ng mL^{-1}) and fentanyl (180 ng mL^{-1})	[81]
Oxycodone and metabolites	LC-QqQ	Hydrolysis: β-glucuronidase Incubation: 60 °C 2.5 h Method 1: LLE (ethyl acetate/n-hexane, 20:80, v/v) Method 2: DUS Method 3: VAMS	(1) 75–86% (2) 76–85% (3) 76–88%	LOD (1): 0.01 ng mL^{-1} LOQ (1): 0.02 ng mL^{-1} LOD (2): 0.20 ng mL^{-1} LOQ (2): 0.50 ng mL^{-1} LOD (3): 0.20 ng mL^{-1} LOQ (3): 0.50 ng mL^{-1}	Comparison of three pre-treatment techniques with microsampling procedures	[51]
60 DOAs including, BZD, opiates, ATS and cathinones	LC-QqQ and LC-QToF	Hydrolysis: β-glucuronidase Incubation: 55 °C 1 h Dilute and shoot	–	–	114 samples: 49 positives in norbuprenorphine, 15 in MOR and 26 in AMP	[36]
Phase I and II metabolites of two synthetic cathinones	LC-QToF	Dilute and shoot (ACN:H_2O, 1:9, v/v) Centrifugation and injection	–	–	Simple dilute and shoot strategy	[31]
Flunitrazepam 7-aminoflunitrazepam	LC-QToF	Extraction: Monolithic C_{18} SPE SpinTip	98–109%	LOD: 0.2–0.5 ng mL^{-1} LOQ: 0.4–1.0 ng mL^{-1}	Novel method with SPE SpinTip extraction	[57]
11 cathinones	LC-Orbitrap	Extraction: SPE (Oasis MCX)	69–125%	LOD: 0.04–0.16 ng mL^{-1} LOQ: 0.2 ng mL^{-1}	Evaluation of two cationic-exchange sorbents in urine	[55]
Fentanyl and analogues	LC-Orbitrap	Extraction: SPE (Nexus)	–	–	84 samples: 48% positive: carfentanyl in 17 samples, fentanyl in 9 and carfentanyl and fentanyl together in 12	[58]
16 DOAs including ATS, opiates and COC	LC-QTrap	Extraction: SPE (In-house mixed-mode)	50–114%	LOD: 3–75 ng mL^{-1} LOQ: 100 ng mL^{-1}	Implementation of a new in-house sorbent	[60]
THC-COOH	LC-QTrap	Incubation: 55 °C 15 min Extraction: LLE (MeOH:ACN, 80:20, v/v)	–	LOD: 3.0 ng mL^{-1} LOQ: 6.0 ng mL^{-1}	35 samples: 27 positives between LOD and 23 ng mL^{-1}	[82]
Morphine and morphine-6-G	CE-FL	Derivatisation: in-CapD (potassium ferricyanide)	85–98%	LOD: 0.5–0.65 ng mL^{-1} LOQ: 1.80–2.25 ng mL^{-1}	Combination of FASI with in-CapD	[85]
Tramadol and MET	CE-UV	Dilution: H_2O	–	LOD: 1.5–2.0 μg mL^{-1} LOQ: 5–7 μg mL^{-1}	Simple and rapid enantiomeric separation	[86]
Mephedrone 4-methylephedrine MDPV	CE-DAD	Extraction: 2 SALLE (toluene)	33–65%	LOD: 15–45 ng mL^{-1} LOQ: 25–100 ng mL^{-1}	Use FASI technique with LLE	[50]
Mephedrone 4-methylephedrine MDPV	CE-DAD	Extraction: 2 LLE (ethyl acetate/IPA) and in-line SPE-CE (Oasis HLB)	65–93%	LOD: 3–8 ng mL^{-1} LOQ: 5–10 ng mL^{-1}	High values of enrichment factor (6000–8000) with SPE-CE	[66]
Mephedrone Methylone 4-methylephedrine MDPV	CE-DAD	Extraction: LLE (cyclohexane)	50–98%	LOD: 4–8 ng mL^{-1} LOQ: 15–20 ng mL^{-1}	The electrokinetic supercharging (EKS) methodology have a high preconcentration potential	[52]
MDPV	CE-MS	Extraction: 2 LLE (ethyl acetate/IPA) and in-line SPE-CE (Oasis HLB)	87%	LOD: 10 ng mL^{-1} LOQ: 30 ng mL^{-1}	First combination of in-line SPE-CE with MS	[49]

mode sorbents, MeOH is usually used. However, these results show that reversed-phase sorbents can also be useful for analysing DOAs in urine.

As for LLE, one important trend in recent years for SPE is miniaturisation. Pérez-Alcaraz et al. [48,49] used an Oasis HLB sorbent for an in-line SPE-CE extraction for a group of synthetic cathinones in urine. An in-line SPE was applied for the first time to chiral CE-MS [49], although a previous LLE was carried out. With this methodology, they achieved a recovery value of 87% for MDPV with an easy enantiomeric separation of the mentioned cathinone. Fujishiro et al. [57] developed a method for determining flunitrazepam and its metabolite by C_{18} SPE SpinTip. This technique combines the SPE process at microscale with a microcentrifugation, and thus reduces the extraction time greatly, achieving recovery

values between 98 and 109%. Moreover, this strategy uses minimal amounts of solvent and also, less sample manipulation is required, so it can be considered as a green strategy. However, and despite of its advantages, as it is a relatively new technique, the limited amount of commercial extracting phases still represents a limitation. Other microextraction techniques reported in the literature based on SPE are μ-SPE, microextraction by packed sorbents (MEPS) and solid-phase microextraction (SPME) [54]. In the case of μ-SPE, Sánchez-González et al. [61,62] synthetized one MIP for determining cannabinoids [61] and another for synthetic cathinones [62]. The μ-SPE device consisted of a cone shape made of a polypropylene porous membrane containing the MIP sorbent, allowing a faster and integrated washing and extraction procedure. They achieved recovery values higher than 86% in both cases, with the main advantage of its high selectivity compared to other sorbents. Malaca et al. [67] used MEPS for determining ATS in urine. They performed an extraction step in less than 3 min, achieving recoveries between 19 and 71%. Other authors, such as Alsenedi et al. [68], extracted a group of ATS and synthetic cathinones using SPME. In their study they compared three different fibres made of PDMS/DVB, C18 and C18-SCX, and found the first of these to be optimal. This method achieved an easy miniaturised extraction, with low solvent and sample consumption. However, the main drawback of SPME is the limited availability of commercial fibres and even that there are different options and also some authors have been developing some selective coatings, more research is still needed in this field.

Over recent years, the combination of microsampling with microextraction has emerged as one of the most used techniques. Dried matrix spots (DMS) and volumetric absorptive microsampling (VAMS) are the most representative strategies. In these approaches, the microsampling and the pre-treatment are carried out in the same collector device and are a combination of three main steps: sampling, drying the device and extracting the analytes [69]. there are several advantages of using these strategies compared to the traditional strategies, including the reduced sample volumes, easy collection, and practical handling, transportation and storage. In addition, it has been proven that better stability of the analytes is achieved when the sample is dried [69]. The DMS technique is based on applying a drop of the matrix to filter paper and then leaving it to dry, whereas VAMS devices are based on a plastic handle and a globous tip that is wicked directly into the matrix to absorb a controlled volume of sample into its pores. Then, the device is dried and extracted for its analysis. VAMS have proved to produce more highly-reproducible dried specimens than DMS since with the first, the volume is fixed, while the spot in the DMS is not controlled [69–71]. In the case of urine specimens, Protti et al. [51] developed a method for evaluating two different procedures that were compared with a LLE for determining oxycodone and its major metabolite. The first procedure was based on urine hydrolysis with β-glucuronidase, incubation and then the dried urine spots (DUS) strategy was applied. For this, the mixture was transferred to a filter paper for drying, extracted with 0.1% HCOOH in MeOH by ultrasound (US) and then with microwave-assisted extraction (MAE). Next, it was evaporated and reconstituted for its injection. The second procedure consisted in the same urine hydrolysis followed by the VAMS strategy. The sampler was a polypropylene rod with a small globous tip, which contacted with the matrix, and it was dried at room temperature for 1 h. Finally, it was extracted with MeOH, sonicated with US, evaporated and reconstituted prior to injection. Both procedures achieved excellent recoveries (higher than 67%), although the obtained values were slightly better for the VAMS technique, which were between 76 and 88%. The main conclusions reported for the authors after comparing LLE, DUS and VAMS were that no differences were observed in terms of recoveries but the use of these microsampling techniques considerably reduced the use of organic solvent and manipulation and also improved the stability of the analytes. In general, it can be concluded that there is not a unique pre-treatment procedure and depending on the purpose and the needs of the analysis, the choice of one pre-treatment over others is crucial. SPE and LLE have been the most used techniques in last years for DOAs determination in urine. These techniques provide, in general, high preconcentration factors, which are very useful to determine these substances at the low levels of concentration at which they are usually in urine. As the last trends are based on green strategies, there has been an increase in the use of microextraction techniques such as DLLME, SPME, MEPS or different μ-SPE strategies. They offer a sample pre-treatment with the use of very low volumes of urine and organic solvent and even that their preconcentration factors are lower than conventional SPE or LLE, with their use in combination with very sensitive techniques as LC-MS, the usual levels at which DOAs are present in urine from drug abusers can be achieved. In the last years, another trend has been the use of sampling techniques able to proceed with extremely low sample volumes. DUS or VAMS strategy have been used for this purpose as they are easy and green strategies. Their main drawback is that as only one spot of urine is used for the analysis, the preconcentration factor is limited and thus, the sensitivity is reduced. Even that further research is needed, they are a promising tool for forensic and toxicological analyses.

2.2. Urine analysis techniques

The most common chromatographic techniques for determining DOAs in urine are LC and GC, being LC the most used. An overview of the use of the chromatographic and related techniques for determining DOAs in urine from 2018 to June 2021 is shown in Fig. 1. GC has been commonly used in recent years for drug determination in urine [39–41,43–47,63–65,67,72–75]. However, it is not as usual as LC because most drugs are not volatile compounds. Therefore, when GC is employed a derivatisation process is usually needed prior to the analysis, which increases the analysis time. The most used derivatisation agents are trifluoroacetic anhydride (TFAA) [43,45,46] and pentafluoropropionic anhydride (PFPA) [44,72,73]. As far as we know, all the methods developed for drug determination in urine using GC in the years reviewed are coupled to MS or MS/MS, and triple quadrupole (QqQ) is the preferred choice for several authors [39–41,43–47,63–65,67,72–75]. Both the LLE [39–41,43–47] and SPE [44,63–65,67,72–75] pre-treatment techniques are widely used in combination with GC. For example, Nisbet et al. [44] developed a method for determining 23 NPS combining LLE and SPE prior to the derivatisation with PFPA and GC-MS analysis. They achieved low levels of ng mL^{-1} for all of the analytes. Kim et al. [45], who developed a methodology for 13 AMP-related NPS by GC-MS, analysed seven urine samples from drug abusers from the Narcotics Department. They found four compounds with AMP levels from 39 to 499 ng mL^{-1}, MAMP between 106 and 621 ng mL^{-1}, and MDMA and its metabolite MDA at 445 and 17 ng mL^{-1}, respectively. Alsenedi et al. [68] determined a group of ATS and cathinone substances in urine by SPME followed by GC-MS. They analysed three previously reported positive samples of cathinone and found concentrations from 227 to 1209 ng mL^{-1}. Alremeithi et al. [75] used a GC-QqQ to enantiomerically determine 14 synthetic cathinones. They performed a SPE followed by the enantiomerical derivatisation with the chiral agent (S)-(−)-N-(trifluoroacetyl)pyrrolidine-2-carbonyl chloride (L-TPC) to obtain cathinone L-TPC derivates. Mercieca et al. [41] developed a method for determining 26 DOAs by GC-MS with a fast derivatisation using hexyl chloroformate in only 30 s of manual shaking.

Urine was hydrolysed and rapidly derivatised for the subsequent DLLME. Although they performed a fast derivatisation, they achieved higher LODs and LOQs than the previous methodologies. Urine samples from autopsies were analysed with the developed method. MDMA was found at concentrations of 9500 and 5000 ng mL^{-1}, and MDA was found at 280 and 50 ng mL^{-1}. These results show the wide range of concentrations at which these substances can be found in urine from drug abusers and the importance of determining both the parent compounds and their metabolites. For example, when MDMA was found, its metabolite MDA was also detected at lower concentrations, which means that MDMA was not fully metabolised and it probably was a recent consumption. Even the good results of sensitivity generally achieved by GC when coupled to MS detectors, its main drawback is related to the need of a derivatisation step due to the polar characteristics of most DOAs, which usually means longer analysis times and also the need of some non-environmentally solvents such as hexyl chloroformate.

As mentioned above, LC is the most employed technique for analysing DOAs in urine [31–34,36,38,47,51,55–58,60–62,64,76–83]. Due to the need for sensitive methods MS plays an important role, and in this sense, LC is mostly coupled to MS/MS, and more precisely to QqQ [32–34,36–38,51,56,59,61,62,76–81]. The methods developed with LC generally offer more sensitivity compared to GC, achieving LODs and LOQs around ten times lower. For example, Di Trana et al. [81] achieved LODs between 0.02 and 0.25 ng mL^{-1} and LOQs between 0.06 and 0.5 ng mL^{-1} in a method that included a total of 77 NPS, 24 classic drugs and 18 metabolites by LC-QqQ. It is worth mentioning that these results were obtained by simply dissolving the urine sample with a commercial M3® buffer solution. With this methodology they found different DOAs in 23 out of 56 urine samples from different individuals. Different compounds, such as COD (14–150 ng mL^{-1}), MOR (58–380 ng mL^{-1}) and fentanyl (0.7–710 ng mL^{-1}), were found in the samples. Pascual-Caro et al. [55] obtained similar results in terms of LODs and LOQs in determining 11 synthetic cathinones by SPE followed by LC-HRMS using Orbitrap as analyser. They achieved LODs between 0.04 and 0.16 ng mL^{-1} and LOQs of 0.2 ng mL^{-1} for all the compounds. The same authors compared LC-HRMS and LC-MS/MS for determining the same group of cathinones [84]. They stated that lower LODs and LOQs were obtained with LC-MS/MS using QqQ as analyser. Al-Asmari et al. [37] also used LC-QqQ for determining THC and its metabolites in urine from autopsies using hydrolysis and SPE in the pre-treatment. They found concentrations of 4 ng mL^{-1} for THC and 224 and 4 ng mL^{-1} for THC-COOH and THC-OH, respectively. Other authors, such as Fujishiro et al. [57], used a LC-QToF for determining flunitrazepam and its metabolite in urine with a SPE SpinTip extraction. This method was applied to urine from a volunteer after oral administration. It could be observed that the maximum concentration of flunitrazepam was achieved after 2 h of the administration (2.38 ng mL^{-1}) and one of its metabolites was obtained after 10 h (102 ng mL^{-1}), which is useful for determining the metabolization of flunitrazepam. Less sensitivity was obtained with LC-QTrap, used by Musile et al. [60], Gerace et al. [82] and Lee et al. [64] compared to Di Trana et al. [81] who included some of the same DOAs as mentioned above. Gerace et al. [43] determined a group of synthetic cathinones by incubation and LLE, and found 81 mg L^{-1} of mephedrone, as well as other cathinones at lower concentration levels. These results show that with a more sensitive instrumentation, such as a LC-QqQ, very low concentration levels of different DOAs and their metabolites can be found in urine samples from drug abusers.

CE has also been used by some authors to determine different types of drugs in urine [48–50,52,85,86], and it is particularly useful for the enantiomeric determination of drugs. This can be easily performed by simply adding a chiral selector to the BGE, and therefore expensive columns, specific organic solvents and long analysis times are not necessary as in the case of LC and GC. The most used chiral selectors are native cyclodextrins (CDs), although ethers, sugars and macrocyclic antibiotics are also employed [87–89]. For example, Naghdi et al. [86] determined tramadol and MET by CE-UV and achieved a rapid chiral separation by simply diluting urine with H_2O and using a chiral selector such as maltodextrin with dextrose.

Despite the mentioned advantages of CE regarding the enantiomeric separation, this technique has lower sensitivity compared to LC and GC. Therefore, two procedures can be carried out to preconcentrate the analytes: one based on chromatographic principles (SPE) and one based on electrophoretic principles. For example, regarding the chromatographic principles, Pérez-Alcaraz et al. [66] developed a methodology for determining three synthetic cathinones by CE-DAD using native CDs as chiral selectors. They performed a LLE followed by the chiral CE separation using in-line SPE-CE with enrichment factors between 6000 and 8000. With these values, they achieved a similar sensitivity as that achieved with other methods based on using LC or GC. Regarding the preconcentration based on electrophoretic principles, the same authors used field-amplified sample injection (FASI) [50] after a SALLE pre-treatment for the enantiomeric determination of three synthetic cathinones in urine, achieving enrichment factors of around 600. The same authors reported another strategy based on the enantiomeric determination of four cathinones, but in this case, they preconcentrated the analytes using LLE and the electrokinetic supercharging (EKS) principle [52], and achieved enrichment factors of around 1000. Zarad et al. [85] performed a preconcentration of the sample using FASI combined with in-capillary derivatisation (in-CapD) of morphine and morphine-6-glucuronide. The derivatisation was performed to produce derivates with a higher fluorescence intensity, and thus enhance the sensitivity of the CE-FL, which achieved fourteen times more signal than without it. Although CE has mainly been used with DAD detection, some authors have employed CE-MS for determining DOAs in urine. In this case, it is important to use low conductivity BGEs, volatile chiral selectors or different strategies to prevent incompatible chiral selectors entering the MS system. When compatible chiral selectors are used, instead of using native CDs, modified charged CDs such as sulphated, phosphated or sulphobutylated CDs can be employed. Moreover, there are two widely used strategies for chiral CE-MS separation: the partial filling technique and counter-current migration technique. These are employed along with charged chiral selectors to avoid these substances entering the MS system [89,90]. For example, Pérez-Alcaraz et al. [49] combined for the first time an in-line SPE-CE with MS detection for determining a synthetic cathinone and using LLE as a sample pre-treatment step. With this methodology, they achieved a rapid enantiodetermination of a cathinone by only using sulphated CD in the BGE. They stated that higher LODs and LOQs were obtained using CE-MS compared to their previous methodology with CE-DAD [66] due to the modifications required to make it compatible for use with MS. As an example, Fig. 2 shows an electropherogram obtained with the in-line SPE-CE methodology developed by Pérez-Alcaraz et al. [49] for the enantiomeric determination of MDPV in urine in less than 20 min.

3. Oral fluid

OF or saliva is a promising alternative to other biological matrices that have been generally considered for drug testing, and in the past decade it has gained more importance [12]. It is used to

detect recent drug consumption, as the drug can be found from minutes to hours after consumption. OF is easy to collect, specialized personal are not necessary and it is a non-invasive and observable collection, so it is difficult to adulterate the sample. Moreover, the parent drug is usually found at higher concentrations than their metabolites, as the drug is not or not totally metabolised. On the other hand, the volume of saliva obtained is low (around 1 mL) and there can be contamination from mouth residues [12,27,28,91].

In general terms, the different steps for OF analysis are sample collection, extraction of the analytes and their determination. Table 2 shows the most recent methodologies developed for determining DOAs in OF by chromatographic and related techniques.

3.1. Of sample collection and pre-treatment

Saliva can be collected by using different strategies, such as aspirating it with passive drool; that is, by using a straw to collect the saliva pooled at the bottom of the mouth. It can also be collected by transferring the saliva to a recipient by spiting or by placing a swab in the oral cavity to absorb the saliva in a short time [92–94]. In Table 2, it can be observed that the most common approaches and the most used in recent years have been direct spitting [81,82,95–104] and using a commercial collector device [96,105–120]. Several sample collection devices have been developed and all of them are based on a small swab or pad that absorbs the saliva, which is then placed in a conical tube to be centrifuged. These devices have a small hole to let the saliva pool at the bottom of the device, where it is finally collected [12,121,122]. One important parameter to study is the amount of substance linked to the collector, as the adsorption of the analytes from the saliva to the swab can be different depending on the type of commercial devices and also on the particular DOAs under study. For example, LSD, MTD or THC experienced problems of linking to the swab and consequently, low recoveries with various collector devices have been observed for the different types [122]. Their main advantages are that they allow a reproducible amount of saliva to be collected because the swab volume is controlled, they are a hygienic strategy and there are also a wide variety of commercial devices available [91,93]. Within these devices, Salivette® [105,112,116,118–120] and Quantisal™ [96,106,107,110,111,115,117] are the most used. The choice of a specific device is a significant variable in the method because its efficiency may be different [92,93,122]. For example, the swab can be produced by different absorptive materials, such as cotton, which is the most common, synthetic fibres or cellulose, among others. Depending on the manufacturer, different collection methods and times may be specified, such as keeping the pad under the tongue or chewing it. Another important aspect is the swab capacity, as different volumes can be collected (normally around 1 mL) [122].

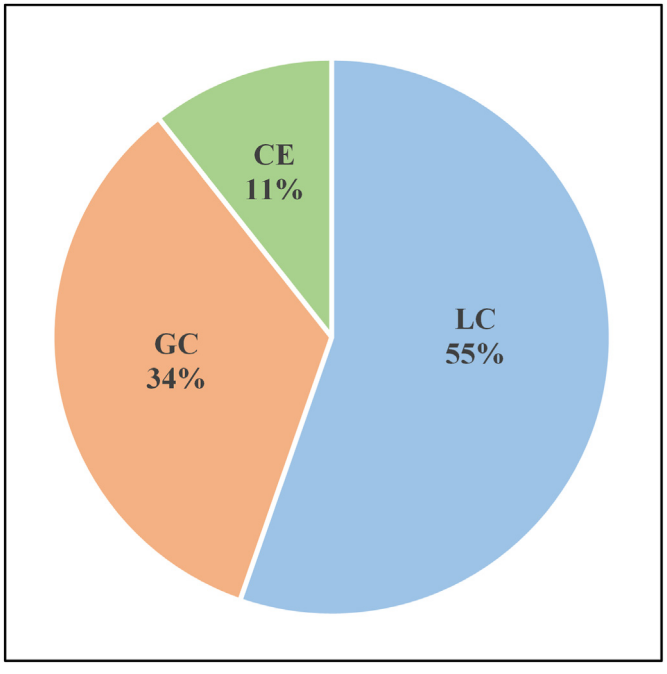

Fig. 1. Application of the different chromatographic techniques for drugs of abuse analysis in urine.

When a swab has been used to take the sample, a desorption of the analytes is necessary and a centrifugation is usually performed to let the saliva pool at the bottom of the collector device. In some cases, a solvent is added in the centrifugation process to facilitate the desorption of the analytes, such as MeOH or Quantisal™ buffer. This buffer is designed for the Quantisal™ collector, although it has also been used with other collection methods [95,103,105,109,111]. A strategy that some authors use is the dilution of the matrix [81,106,117]. For instance, Krotulski et al. [117] collected the saliva with a Quantisal™ device, centrifugated it and diluted the saliva with the Quantisal™ buffer to determine 12 DOAs. With this simple procedure, they achieved recovery values between 87 and 106%. Other authors also used the dilute and shoot strategy for different types of DOAs in OF after its collection by direct spitting. In this case, recoveries higher than 77% with a minimal sample pretreatment were achieved [106]. Since OF is not an extremely complex matrix as for example serum or plasma, a straightforward dilution of the OF was performed without any additional pretreatment, so the need of a time-consuming and expensive pretreatment was not required. However, dilute and shoot strategy simply decreases the matrix effects and when high sensitivity is needed, the use of this unique strategy can be problematic.

Therefore, in some cases, after the desorption from the swab, a treatment step is applied to clean the sample and pre-concentrate the analytes. For this purpose, LLE has been a very common strategy for some authors and different solvents have been employed, such as MeOH, ACN, H_2O, heptane, or mixtures of them [82,97,101,102,109,116]. For example, Duan et al. [102] collected the OF by direct spitting and performed a LLE with diethyl ether, achieving recoveries between 45 and 105%. Moreover, Da Cunha et al. [107] also added a salt to perform a SALLE, using this technique and the Quantisal™ collector for determining 104 NPS. In this procedure, they used methyl *tert*-butyl ether (MTBE) for the extraction, obtaining recovery values from 8 to 113%. Although very low recovery values were achieved for some NPS, most of them were higher than 60%, and it should be highlighted that an important achievement of this method is the large number of DOAs included. Other authors have used the miniaturisation and the consumption of low volumes of organic solvent with techniques such as DLLME [111,112]. This is the case of Fernández et al. [112], who used a Salivette® device for the sampling and after centrifugation and a protein precipitation, they performed a US-DLLME with chloroform. In this strategy, the protein precipitation was performed with MeOH, that was used as protein precipitant and as dispersant solvent. They obtained recoveries from 74 to 129% for all the compounds; however, they used a non-environmentally friendly solvent: chloroform. It is important to mention that due to the limited OF volume collected, LLE and DLLME generally uses for this matrix low volumes of sample (1 mL or less) and similar volumes of organic solvent. For that reason, high preconcentration

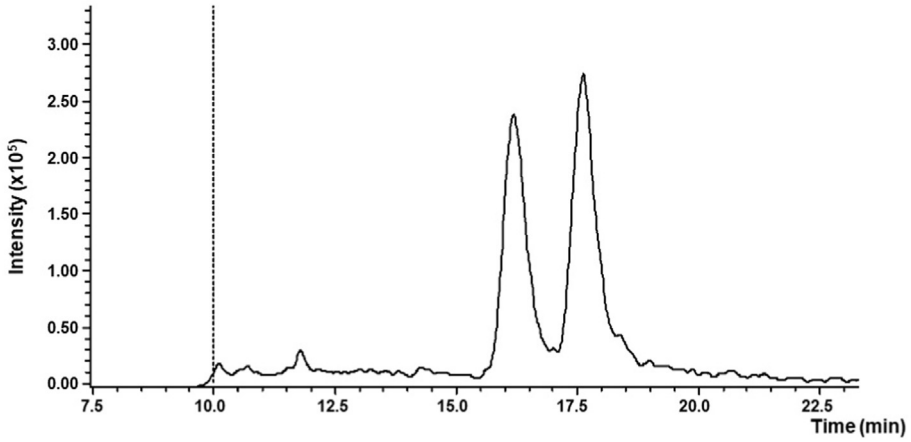

Fig. 2. An electropherogram of the in-line SPE-CE method developed by Pérez-Alcaraz et al. for the enantiomeric determination of MDPV in urine. Reproduced with permission from ref. [49].

factors are not achieved with LLE but less organic solvent is used.

Despite the wide use of LLE, the most employed extraction technique for the pre-treatment of samples is SPE [95,96,99,100,103,109,113,115,116]. Similarly to urine, the most common SPE sorbents are mixed-mode cationic-exchange sorbents due to the basic characteristics of most DOAs [95,96,99,103,109,115]. For example, Borg et al. [115] used a Strata-XC SPE sorbent for the chiral determination of MAMP and employed a Quantisal™ device for the sample collection. They also performed a simple derivatisation process using Marfey's reagent, consisting of adding a small volume of the reagent to the extracted solvent and heating it for 1 h to obtain the two enantiomers. With this procedure they achieved recovery values of 87 and 89%. In this case, they avoid using special columns for the enantioseparation by LC.

Some authors have also used a combination of LLE followed by SPE to increase the process efficiency [109,116]. For example, Fabresse et al. [109] collected the saliva with a FLOQSwab™ device for determining 10 DOAs. The swab was introduced into Quantisal™ buffer and sonicated and a LLE with heptane was performed. The LLE solution was analysed for detecting THC and for the other DOAs, a SPE with a cationic mixed-mode sorbent (Drug-X-B) was carried out. With this methodology, they achieved recoveries of between 19 and 55% for all the compounds. Wang et al. [116] also performed a SPE procedure after the LLE using a reversed-phase cartridge as the Bond Elut C_{18}, obtaining recovery values between 86 and 109% for five BZD.

Other authors such as Sorribes-Soriano et al. [98] developed a pipette-tip extraction using a synthetic polymerised monolithic sorbent. This was the first time that a monolith in-tip was used for determining DOAs in OF samples. They collected the sample by direct spitting and after the pre-treatment, they obtained recoveries from 64 to 115% for 22 NPS.

Other strategies used for extracting DOAs in OF samples are incubation [110], supported liquid extraction (SLE) [114], MEPS [123] and SPME [83]. For example, Desharnais et al. [110] collected the OF with a Quantisal™ device for determining 97 DOAs, and then the sample was incubated for 72 h. With this methodology, they achieved recoveries higher than 80% for all the compounds. Bruun et al. [114], who determined oxazepam and zopiclone, collected the OF using the Intercept® device. After a centrifugation, the solution was transferred onto a SLE plate to absorb the analytes. the entire SLE procedure was automated and completed in 1.5 h. Bianchi et al. [123] developed an automated MEPS methodology to determine NPS in OF, achieving recovery values from 83 to 120% and collecting the sample by direct spitting. Anzillotti et al. [83] also collected the sample by spitting and employed a SPME-GC/MS method for determining synthetic and natural cannabinoids in OF using PDMS as fibre material and reducing the analysis time and solvent consumption.

In an attempt to simplify the procedure, some authors did not perform an extraction step after the sample collection [105,118–120]. For example, Pascual-Caro et al. [105] developed a method for determining 11 synthetic cathinones in OF. The only pre-treatment necessary was to desorb the analytes from the Salivette® twice with MeOH and evaporate the combined extract. With the developed methodology, they achieved an easy and fast method for determining cathinones using small volumes of MeOH as organic solvent, with recoveries from 50 to 66% for all the analytes. Moreover, they studied the effect of discarding or keeping the first centrifugate and concluded that the recoveries were similar, and if the first centrifugate was discarded, the matrix effect was considerably reduced (up to 13%).

Regarding the latest trends in using DMS and combining the sampling with the pre-treatment, dried OF spots (DOFS) have been employed by some authors for determining DOAs in saliva [104,124,125]. This strategy makes it possible to use a very small amount of sample, which is collected by spitting into a polypropylene tube. It is dried before storage using filter paper. This method is very useful not only for sampling, but also for preserving the sample. Once the sample is dried in the paper, a solvent is added to desorb the analytes with the use of sonication or centrifugation. For example, Gorziza et al. [125] used this strategy for determining 5 DOAs, achieving recoveries from 54 to 84%. For the extraction, they only used 1 mL of a mixture of MeOH and ACN with sonication and centrifugation. Therefore, they obtained an environmentally friendly technique and better analyte stability, which is interesting from the toxicological point of view for possible analysis repetitions.

Even that several sample pre-treatments have been used in the last years for OF, there is not a universal sample preparation technique and their choice depends on different factors as the type of analytes or the final purpose of the analysis. SPE and LLE have been the two most used extraction techniques for OF analysis in the toxicological and forensic field in last years. However, these techniques suffer from several drawbacks as being time-consuming or requiring the consumption of relatively large amounts of organic solvents. In this regard, microextraction techniques have been

Table 2
Analytical methodologies for the determination of drugs of abuse in oral fluid samples.

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
12 cannabinoids	GC-MS	Collection: not specified Extraction: DI-SPME	–	LOD: 1–10 ng mL^{-1} LOQ: 1–10 ng mL^{-1}	106 samples: 4 positive in THC (10–655.2 ng mL^{-1}), CBD (4.5–15.3 ng mL^{-1}) and CBN (66.2 ng mL^{-1})	[83]
Tramadol and MTD	GC-MS	Collection: not specified Extraction: Magnetic extraction with synthetized layered double hydroxide	87–90%	LOD: 0.5–0.8 ng mL^{-1}	Easy magnetic separation and large adsorption capacity with the nanocomposite adsorbent	[74]
KET, mephedrone and synthetic cannabinoids	GC-MS	Collection: not specified Extraction: MEPS	83–120%	LOQ: 0.05–0.5 mg L^{-1}	Rapid screening with MEPS in combination with desorption electrospray ionisation mass spectrometry (DESI-HRMS)	[123]
MTD EDDP	GC-QqQ	Collection and extraction: DOFS	45–74%	LOD: 5 ng mL^{-1} LOQ: 10 ng mL^{-1}	Routine analysis: MTD (246.6 ng mL^{-1}) and EDDP (20.6 ng mL^{-1})	[104]
11 cathinones	LC-QqQ	Collection: Salivette® Extraction: MeOH and centrifugation	50–66%	LOD: 0.003–0.030 ng g^{-1} LOQ: 0.075 ng g^{-1}	Pre-treatment in the same collector device	[105]
20 DOAs including cathinones, MOR, COC, MTD and naloxone	LC-QqQ	Collection: Salivette® Protein precipitation: MeOH Extraction: US-DLLME (chloroform)	74–129%	LOD: 0.1–25 ng mL^{-1} LOQ: 0.25–50 ng mL^{-1}	15 samples: MOR (875.8–8323.7 ng mL^{-1}), COC (2.0–12529.3 ng mL^{-1})	[112]
5 ATS	LC-QqQ	Collection: Quantisal™ Dilution: Quantisal™ buffer Extraction: DLLME	–	LOQ: 20 ng mL^{-1}	140 samples: MAMP (31.5–248.1 ng mL^{-1}) and MDMA (21.6–200000 ng mL^{-1})	[111]
97 DOAs	LC-QqQ	Collection: Quantisal™ Incubation: acetone:ACN, 30:70,*v/v*)	>80%	–	A second injection is performed for CBD	[110]
R/S MAMP	LC-QqQ	Collection: Quantisal™ Dilution: phosphate buffer Extraction: SPE (Strata-XC) Derivatisation: Marfey's	87–89%	LOQ: 2.5 ng mL^{-1}	251 positives of S-MAMP (3820 ng mL^{-1}). R-MAMP (984 ng mL^{-1})	[115]
17 DOAs	LC-QqQ	Collection: Quantisal™ Extraction: dilute and shoot. Centrifugation and injection	77–115%	LOQ: 0.5–1 ng mL^{-1}	Minimal samples preparation	[106]
104 NPS	LC-QqQ	Collection: Quantisal™ Extraction: SALLE (MTBE)	8–113%	LOD: 0.05–10 ng mL^{-1}	7 positives finding 16 NPS	[107]
10 DOAs including MOR, 6-AM and buprenorphine	LC-QqQ	Collection: direct spitting and Quantisal™ Dilution: phosphate buffer Extraction: SPE (PolyChrom Clin II)	51–95%	LOD: 5 ng mL^{-1} LOQ: 10 ng mL^{-1}	18 samples: 4 positives of MOR (32–146 ng mL^{-1}) and 3 in 6-AM (15–110 ng mL^{-1})	[96]
37 DOAs including ATS, BZD and opiates	LC-QqQ	Collection: Oral-Eze Precipitation: ACN and MeOH	45–111%	LOQ: 0.5 ng mL^{-1}	10 samples: AMP (271 ng mL^{-1}), COD (840 ng mL^{-1}) and THC (23 ng mL^{-1})	[108]
10 DOAs	LC-QqQ	Collection: FLOQSwabs™(FS) Dilution: Quantisal™ buffer Extraction: USE. LLE (heptane) and SPE (Drug-X-B)	19–55%	LOD: 1–10 ng mL^{-1}	THC only needed LLE. Variability in OF volume	[109]
Oxazepam Zopiclone	LC-QqQ	Collection: Intercept OF Drug Test Extraction: SLE (ethyl acetate/heptane)	–	LOQ: 0.10 nmol L^{-1}	Development of an experimental design with long periods of sample collection	[114]
77 NPS, 24 classic drugs and 18 metabolites	LC-QqQ	Collection: direct spitting Dilution (M3® buffer)	>80%	LOD: 0.03–0.25 ng mL^{-1} LOQ: 0.07–0.8 ng mL^{-1}	14 samples: COC (28 ng mL^{-1}), MOR (0.5 ng mL^{-1}) and butylone (40 ng mL^{-1})	[81]
24 DOAs including cathinones and cannabinoids	LC-QqQ	Collection: direct spitting Extraction: SPE (Bond Elut Certify)	75–113%	LOQ: 0.25–1.0 ng mL^{-1}	SPE procedure for both cathinones and cannabinoids	[95]
15 natural and synthetic cannabinoids	LC-QqQ	Collection: direct spitting Extraction: LLE (MeOH)	–	LOD: 1.0–2275 ng mL^{-1} LOQ: 3.3–7583 ng mL^{-1}	Future studies to reduce matrix effect	[97]
7 opiates	LC-QqQ	Collection: direct spitting Extraction: SPE (CEREX® Clin II)	–	LOD: 0.01–0.04 ng mL^{-1} LOQ: 0.4–1.5 ng mL^{-1}	17 samples: COD (6.0–122.6 ng mL^{-1}) and hydrocodone (1.9–319.9 ng mL^{-1})	[99]
22 NPS	LC-QqQ	Collection: direct spitting Extraction: pipette-tip extraction with synthetic sorbent	64–115%	LOD: 0.03–0.6 ng mL^{-1}	Innovative microextraction technique with home-made sorbent	[98]
32 DOAs including ATS, cathinones and phenethylamines	LC-QqQ	Collection: direct spitting Extraction: SPE (MIP)	80–120%	LOD: 0.03–1.3 ng mL^{-1}	A MIP based on MAMP was synthetized with high recoveries for different compounds	[126]
AMP MAMP KET BZE Mitragnine	LC-QqQ	Collection and extraction: DOFS	54–84%	LOQ: 0.2 ng mL^{-1}	Comparison of DOFS and WAX-S tip extraction (combination of SPE and SALLE). Interesting to study both in parallel	[125]
CBD THC	LC-QqQ	Collection and extraction: DOFS	24–29	LOD: 2–4 ng mL^{-1}	Comparison of DOFS with pipette-tip extraction	[128]
550 NPS		Collection and extraction: DOFS	–	–	229 samples analysed. NPS detected in 17 of them	[129]

Table 2 (*continued*)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
	LC-QqQ and LC-HRMS					
5 BZD	LC-QTrap	Collection: Salivette® Extraction: LLE (H_2O) and SPE (Bond Elut C_{18})	86 −109%	LOD: 0.01−0.2 ng mL^{-1} LOQ: 0.05−0.5 ng mL^{-1}	Detection time of 15 days of diazepam and nordiazepam in OF	[116]
CBD THC	LC-QTrap	Collection: direct spitting Extraction: LLE (MeOH:ACN, 80:20,v/v)	–	LOD: 0.5 ng mL^{-1} LOQ: 1.0 ng mL^{-1}	CBD (6−18 ng mL^{-1})	[82]
19 cannabinoids	LC-QTrap	Collection: direct spitting Extraction: LLE (H_2O and ACN)	–	LOD: 1 ng mL^{-1} LOQ: 2.5 ng mL^{-1}	12 samples analysed. No cannabinoids found	[101]
Selegiline Desmethylselegiline R/S AMP R/S MAMP	LC-QTrap	Collection: direct spitting Extraction: LLE (buffer and diethyl ether)	46 −105%	LOD: 0.1−0.5 ng mL^{-1} LOQ: 0.2−1.0 ng mL^{-1}	Selegiline (0.5 ng mL^{-1}), desmethylselegiline (0.7 ng mL^{-1}), R-MAMP (61.7 ng mL^{-1}), R-AMP (21.2 ng mL^{-1})	[102]
12 DOAs including cathinones and MDMA	LC-QToF	Collection: Quantisal™ Dilution: Quantisal® buffer	87 −106%	LOD: 1 ng mL^{-1} LOQ: 4 ng mL^{-1}	352 positives: MDMA (4−10000 ng mL^{-1}), α-PVP (87.8−1301 ng mL^{-1}) and methylone (40.3 −7795 ng mL^{-1})	[117]
Non-target	LC-QToF	Collection: Greiner Bio-One Extraction: SPE (Strata-X)	–	LOD: 1.0−100 ng mL^{-1}	59 samples: MTD (29), EDDP (25), diazepam (16), COC (13), AMP (5), MAMP (5) and THC (6)	[113]
14 fentanyl analogues	LC-QToF	Collection: direct spitting Dilution: Quantisal™ and phosphate buffer Extraction: SPE (CEREX® Clin II)	71 −117%	LOD: 0.25−2.5 ng mL^{-1}	20 samples: No fentanyl positives. Other DOAs detected	[103]
COC BZE Cocaethylene AMP MDMA	LC-MS	Collection and extraction: DOFS	–	LOQ: 40 ng mL^{-1}	Simple and fast DOFS extraction	[124]
13 DOAs	CE-FL	Collection: Salivette® Extraction: LLE (ACN)	–	LOD: 0.5−9500 ng mL^{-1} LOQ: 1.6−31670 ng mL^{-1}	Portable CE apparatus. Finding AMP, MDA, MDMA, COC and cocaethylene in real OF samples	[118]
7 DOAs including ATS and COC	CE-FL	Collection: Salivette® Extraction: LLE (ACN)	11−35%	LOD: 6−40 ng mL^{-1} LOQ: 10−66 ng mL^{-1}	128 analyses with the portable CE	[120]
MDMA MDA MDEA	CE-FL	Collection: Salivette® Extraction: LLE (ACN)	<50%	LOD: 3.3−3.8 ng mL^{-1} LOQ: 5.5−6.4 ng mL^{-1}	MDMA (137−7126 ng mL^{-1})	[119]

developed and successfully used for OF samples in last years, with the aim of developing faster and more environmentally friendly sample preparation protocols. For example, the use of strategies as DLLME, SPME or pipette-tip extraction, have resulted in lower requirements for sample amount or lower solvent consumption and increase of the speed of the analytical method. Moreover, these techniques are in compliance with environmental friendly methodologies, and even that the sensitivity usually achieved with these procedures is usually lower than with conventional SPE or LLE, they are useful to determine DOAs in OF because in this sample, these analytes are usually present at high levels of concentration. In addition, there are some studies in which DOFS have also been applied and even that it is still a novel technique and further research is needed, the results reported until now are very promising.

3.2. Of analysis techniques

As with urine, LC and GC are the most employed techniques for determining DOAs in OF, and LC was the most usual technique between 2018 and June 2021 (Fig. 3). Different GC methodologies have been reported for OF samples in recent years focused on compounds that do not need a derivatisation step [74,83,104,123]. As far as we know, all GC methods developed for OF are coupled to MS or MS/MS [74,83,104,123]. It has also been observed that different pre-treatment techniques have been used with GC, such as SPME [83], magnetic extraction [74], MEPS [123] and DOFS [104]. For example, Adlnasab et al. [74] performed a magnetic extraction based on a layer made of a nanocomposite adsorbent prior to its analysis by GC-MS for determining tramadol and MTD. With this method, they achieved LODs 10 times lower than Ribeiro et al. [104], who performed a DOFS strategy for the same compounds followed by GC-QqQ. In this last strategy, the authors applied the method to OF samples from opiate addicts and found concentrations of MTD and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) of 246.6 and 20.6 ng mL^{-1}, respectively. In the case of cannabinoids, Anzillotti et al. [83] found four positive OF samples with concentrations from 10.0 to 655.2 ng mL^{-1} for THC, from 4.5 to 15.3 ng mL^{-1} for cannabidiol (CBD) and 66.2 ng mL^{-1} for cannabinol (CBN) by using DL-SPME as pre-treatment prior to the GC-MS analysis. These results show that GC is suitable for determining these compounds at the usual concentrations that they are found in OF samples from drug abusers.

Despite the good results obtained with GC, LC is the most used technique for determining DOAs in OF [81,82,95−99,101−103,105−117,124−126]. Most of the authors couple LC to MS/MS and more precisely to QqQ [81,95−99,105−112,114,115,125,126], although LC-QToF [103,113,117] and LC-QTrap [82,101,102,116] have also been used by some authors. In general terms, lower LODs and LOQs have been obtained using LC-QqQ. Pascual-Caro et al. [84] compared this instrumentation with LC-HRMS for determining cathinones in OF, using only a Salivette® device to perform the pre-treatment in the same collector. They concluded that the LC-QqQ provided slightly better sensitivity, achieving MDLs two times lower for some compounds [105]. Di Trana et al. [81] also used LC-QqQ for determining 77 NPS, 24 classic drugs and 18 metabolites by diluting the sample after its collection by direct spitting, and achieved LODs between 0.03 and 0.25 ng mL^{-1} and LOQs from 0.07 to 0.8 ng mL^{-1}. They analysed 14 saliva samples from drug abusers and found COC (28 ng mL^{-1}), MOR (0.5 ng mL^{-1}), butylone (40 ng mL^{-1}) and carfentanyl, which could only be identified. Reinstadler et al. [113] also determined a large number of DOAs, but in this case with

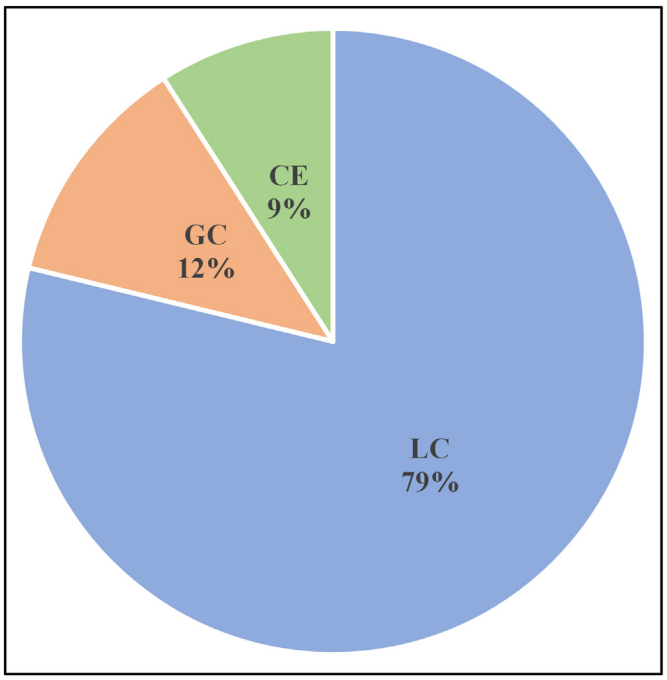

Fig. 3. Application of the different chromatographic techniques for drugs of abuse analysis in oral fluid.

a LC-QToF and SPE with Strata-X cartridge pre-treatment. Although a SPE procedure was performed, the LODs achieved (from 1 to 100 ng mL^{-1}) were higher than those previously reported by Di Trana et al. [81], but in that case, a non-target methodology was performed with the LC-QToF. Reinstadler et al. [113] also applied the developed methodology to OF samples from opiate addicts. They mainly found MTD and its metabolite EDDP followed by diazepam and COC, among others. Zheng et al. [108] found AMP (271 ng mL^{-1}, COD (840 ng mL^{-1}) and THC (23 ng mL^{-1}) in 10 positive OF samples from drug abusers using LC-QqQ and a protein precipitation as pre-treatment in two steps, first with ACN and then with MeOH, which achieved a better precipitation. Krotulski et al. [117] diluted the sample and used an LC-QToF for determining 12 DOAs in OF from MDMA users. They found concentrations of MDMA between 4 and 10000 ng mL^{-1}, α-PVP from 87.8 to 1301 ng mL^{-1} and methylone between 40.3 and 7795 ng mL^{-1}. Other authors, such as Borg et al. [115], performed a derivatisation process using Marfey's reagent for the enantiomeric separation of R and S MAMP by LC-QqQ. This procedure allows the enantiomeric determination of MAMP without the need for a chiral column and only performing a simple derivatisation process. They analysed previously positive MAMP OF samples that were analysed without chiral separation. They found concentrations of S-MAMP and R-MAMP of around 3820 ng mL^{-1} and 984 ng ml^{-1}, respectively. A chromatogram of the developed method for the enantiodetermination of MAMP is shown in Fig. 4. Although a derivatisation was performed, a rapid chiral separation was achieved, avoiding the use of chiral columns.

CE has also been used for determining DOAs in OF. Saar-Reismaa et al. [118–120] developed a method using a portable CE instrumentation with a FL detector, which was applied to three studies focused on the in-situ determination of different DOAs in OF. The method consisted of collecting the sample with a Salivette® device and desorbing the analytes with ACN. The first study was focused on 13 DOAs, and found AMP, MDMA, MDA, COC and cocaethylene in some samples [118]. The second study determined MDMA and two metabolites, and found MDMA concentrations between 137 and 7126 ng mL^{-1} [119]. The third was focused on improving the LODs and LOQs of some of the DOAs in the first study (AMP and methadone) by modifying the emission filter, which increased the analysis time.

4. Conclusions

Determining DOAs in biological samples such as urine and OF is of interest for the scientific community. DOAs are very popular substances all around the world and every year new compounds continuously emerge onto the illegal market. Therefore, it is important to develop methodologies for determining several types of DOAs in these biological samples to be able to know the consumption pattern of the population. This review compiles and summarizes the most recent strategies in determining DOAs and their metabolites in urine and OF samples using chromatographic and related techniques. In general, the methods developed usually include three main steps: sampling, pre-treatment and analysis. In

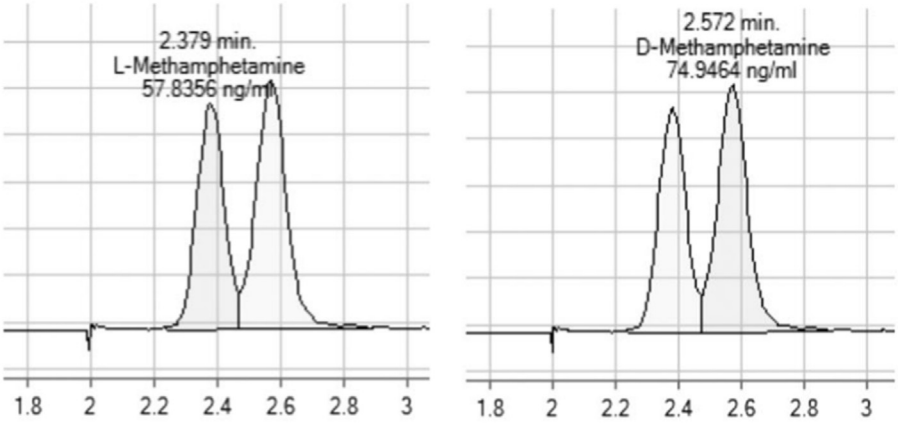

Fig. 4. OF analysed with the developed method by Borg et al. using Marfey's reagent for the derivatisation of MAMP to produce both enantiomers. Reproduced with permission from ref. [115].

the pre-treatment step, LLE and SPE are the most common techniques for removing or reducing the presence of matrix interferences, or for preconcentrating the analytes of interest. However, in recent years novel techniques, such as microextraction techniques have increased in popularity, mainly to obtain more environmentally friendly approaches. Apart from the miniaturised techniques derived from conventional SPE or LLE, DMS have emerged as one of the most promising strategies for their combination of sampling with sample pre-treatment in a miniaturised device. It has been also observed that it is important to determine not only the DOAs but also their metabolites to obtain more information about a drug consumption. In the analysis step, LC and GC combined with tandem MS or HRMS have been the most used techniques to reach the concentration levels at which DOAs are usually present in biological matrices, although LC is preferred due to the polar characteristics of the analytes. However, other approaches based on CE, applied to the chiral separation of some DOAs, are also found in the literature. As new DOAs are constantly appearing and due to the polyconsumption of these substances by drug users, the last trends in the development of new methodologies are based on the process miniaturisation and automatization, the reduction of analysis times and organic solvents and the improvement of sensitive methodologies able to determine a large number of substances.

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.

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