DETERMINATION OF DRUGS OF ABUSE IN URINE AND ORAL FLUID SAMPLES

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UNIVERSITAT ROVIRA i VIRGILI

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DETERMINATION OF DRUGS OF ABUSE IN URINE AND ORAL FLUID SAMPLES

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WE STATE:

That the present Doctoral Thesis entitled "DETERMINATION OF DRUGS OF ABUSE IN URINE AND ORAL FLUID SAMPLES", presented by SERGI PASCUAL CARO for the award of the degree of Doctor, has been carried out under our supervision at the Department of Analytical Chemistry and Organic Chemistry of this university and that all the results presented have been obtained from the experience of the mentioned Doctor.

For the record, we sign this document in Tarragona at 3rd June 2022.

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Ja la tenim aquí! Després d'un treball dur i d'algun moment de patiment em sento molt orgullós d'haver acabat aquest viatge que va començar fa uns quants anys. Han passat moltes coses durant aquest temps, algunes que mai ningú hagués imaginat com una pandèmia o fins i tot una guerra, però sobretot han passat molts bons moments i persones a les quals he d'agrair tot el seu esforç, implicació i recolzament.

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"Cada dia sabem més i entenem menys"

Albert Einstein

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ABSTRACT

[ENG] Drugs of abuse have been present in our lives for years, being amphetamines, cocaine, opioids and cannabis some of the most widely used by the population. In recent times, new psychoactive substances have emerged as an alternative to conventional drugs, and the determination of all these substances is necessary in various biological samples such as urine, saliva, blood or hair. However, as they are complex matrices, once collected, it is very common to use different pre-treatment techniques before analysis. Trends in toxicological and forensic analysis are based on the acquisition of fast methods, which reach low levels of concentration and include the analysis of different types of substances as well as their metabolites.

It is for this reason that this Doctoral Thesis focuses on the development and application of new methodologies for determining different families of drugs, as well as some of their metabolites in urine and saliva samples by liquid chromatography and capillary electrophoresis. The use of the latter technique has been used to determine the enantiomers of some drugs. This fact is important because many drugs are chiral and can have two enantiomeric forms with different pharmacokinetic and pharmacological properties. The coupling of liquid chromatography and capillary electrophoresis with mass spectrometry achieves high sensitivity. However, due to the low concentrations at which these compounds can be found in biological samples, and that there are often some compounds that can interfere with the analytes of interest, the use of various pre-treatment techniques such as SPE has been a common practice. The applicability of the developed methodologies has been realized by determining drugs of abuse in patients starting a detoxification program.

[CAT] Des de fa anys les drogues d'abús estan presents en les nostres vides, essent les amfetamines, la cocaïna, els opioides i el cànnabis algunes de les més àmpliament consumides per la població. En els darrers temps, les noves substàncies psicoactives han emergit com una alternativa a les drogues clàssiques i la determinació de tots aquests tipus de substàncies es fa necessària en diferents mostres biològiques com l'orina, la saliva, la sang o el cabell. No obstant, com que són mostres complexes, un cop col·lectades és molt habitual l'ús

de diferents tècniques de pretractament abans de la seva anàlisi. Les tendències en l'anàlisi toxicològica i forense es basen en l'obtenció de mètodes ràpids, que arribin a baixos nivells de concentració i que incloguin l'anàlisi de diferents tipus de substàncies així com també els seus metabòlits.

És per aquest motiu que la present Tesi Doctoral se centra en el desenvolupament i aplicació de noves metodologies per determinar diferents famílies de drogues, així com també alguns dels seus metabòlits en mostres d'orina i saliva mitjançant la cromatografia líquida i electroforesi capil·lar. L'ús d'aquesta darrera tècnica ha estat utilitzada per tal de determinar els enantiòmer d'algunes drogues. Aquest fet és important ja que moltes drogues són quirals i poden tenir dues formes enantiomèriques amb diferents propietats farmacocinètiques i farmacològiques. L'acoblament de la cromatografia de líquids i l'electroforesi capil·lar amb l'espectrometria de masses permet assolir una alta sensibilitat. Tot i això, a causa de les baixes concentracions a les què aquests compostos es poden trobar en mostres biològiques, i que sovint hi ha alguns compostos que poden interferir amb els analits d'interès, l'ús de diverses tècniques de pretractament com l'SPE és una pràctica habitual. L'aplicabilitat de les metodologies desenvolupades s'ha realitzant determinant drogues d'abús en pacients començant un tractament de desintoxicació.

[ESP] Desde hace años las drogas de abuso están presentes en nuestras vidas, siendo las anfetaminas, la cocaína, los opioides y el cannabis algunas de las más consumidas por la población. En los últimos tiempos, las nuevas sustancias psicoactivas han emergido como alternativa a las drogas clásicas y la determinación de estos tipos de sustancias se hace necesaria en diferentes muestras biológicas como la orina, la saliva, la sangre o el cabello. Sin embargo, al ser muestras complejas, una vez colectadas es muy habitual el uso de diferentes técnicas de pretratamiento antes de su análisis. Las tendencias en el análisis toxicológico y forense se basan en la obtención de métodos rápidos, que lleguen a bajos niveles de concentración y que incluyan el análisis de diferentes tipos de sustancias y sus metabolitos.

Es por este motivo que la presente Tesis Doctoral se centra en el desarrollo y aplicación de nuevas metodologías para determinar diferentes familias de drogas, así como algunos de sus metabolitos en muestras de orina y saliva mediante la cromatografía líquida y electroforesis capilar. El uso de esta última técnica ha sido utilizada para determinar los enantiómeros de algunas drogas. Este hecho es importante ya que muchas drogas son quirales y pueden tener dos formas enantioméricas con diferentes propiedades farmacocinéticas y farmacológicas. El ensamblaje de la cromatografía de líquidos y la electroforesis capilar con la espectrometría de masas permite alcanzar una alta sensibilidad. Sin embargo, debido a las bajas concentraciones a las que estos compuestos se pueden encontrar en muestras biológicas, y que a menudo hay algunos compuestos que pueden interferir con los analitos de interés, el uso de diversas técnicas de pretratamiento como la SPE es una práctica habitual. La aplicabilidad de las metodologías desarrolladas se ha realizado determinando drogas de abuso en pacientes comenzando un tratamiento de desintoxicación.

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CHAPTER 1. INTRODUCTION

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1.1. Drugs of abuse

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One of the main social problems spread all over the world that affects all kind of societies, social classes or cultures is the consumption of drugs of abuse (DOAs). These substances can be very harmful not only for human health but also they can have very negative social consequences, either individual or global. Not all drugs classified as DOAs are illicit substances, some of them as tobacco or alcohol are abused, even that their distribution and sale is allowed [1,2].

The term DOA means that it has to be consumed in sufficient amounts to produce hazard to health or safety, it has illegitimated drug channels and it is taken on individual's own and not medically prescribed. DOAs are psychoactive or psychotropic substances that stimulate the central nervous system modifying the behaviour and the conscience producing dependence and addiction [3].

The drug market is suffering constant changes on its demand as every year the people who consume DOAs is increasing and the interest is to continuously satisfy the drug consumers. Due to the present advances in the new technological era, these changes are focused on new types of drugs, new trafficking methods, new routes of distribution and the increase of online markets [4]. For all these reasons and also because of the important impact on the society caused by DOAs consumption, different organizations were created to control these substances over the world [4,5]. In this regard, the leading authority on illicit DOAs in the European Union (EU) is the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), which was formally established in 1993. Its main aim is to give support to EU and national policymaking by providing evidence-based information on drugs, drug addiction and their consequences. Its work contributes to EU and national policies to protect the citizens from drug-related harms [6]. Since its creation, this European organization has placed particular emphasis in collaborating with other organisms such as the United Nations Office on Drugs and Crime (UNODC), which operates in all regions of the world through an extensive network of field offices. UNODC has as main objective offering practical help to make a safer world from drugs, crime, corruption and terrorism. Its actions to help the drug problem are implementing international drug control treaties and drug use prevention strategies, support drug dependence treatment and rehabilitation, ensure the access to controlled medical substances and analysing and reporting the trafficking trends [7]. Apart from these, the International Narcotics Control Board (INCB) is the responsible of implementing

drug control conventions in the United Nations. INCB also ensures the adequate drug supplies for medical and scientific purposes and control the illicit trafficking and manufacturing [8]. In the United States (US), the Drug Enforcement Administration (DEA) is responsible of combating drug trafficking and manufacturing. They organize the national drug intelligence program to collect and analyse operational drug information and seizure illicit drugs to avoid their trafficking [9]. For example, in these routes to transport DOAs, Spain is one of the most important countries for drug entry to EU for its strategical geographical location. In general, most of the DOAs arrive to Spain from Central or South American countries. For that reason, Spanish law enforcements agencies apply intensive controls in the coastal areas and harbours to identify and dismantle the drug networks [10].

Due to the continuous increasing number of drugs, it is very difficult to be able to control all of them. The main problem observed by the organizations responsible of developing drug policies for DOAs was that the process to control a drug was extremely long, so by the time a drug was controlled another one appeared into the market. For that reason, the EMCDDA improved the overall process and achieved faster regulations focused on reducing the times of controlling a substance, such as establishing the rules to define criminal sanctions, including new policies regarding the illegal market or achieving cooperation in judicial criminal matters. Even though, despite of improving the procedure of controlling a drug, the times were still long [4,11–13]. For that reason, in order to provide rapid information that could result in immediate responses to reduce risk to public health, Early Warning Systems (EWS) were implemented [14]. The main objectives of the EWS are the early detection of new drugs (emerging drugs), the assessment of risks related to the presence of this kind of substances and the establishment of early warnings to produce effective and real-time responses. Their early detection is based on laboratory caseworks from seizures, poisonings and tests from unknown samples. Then, once the substance has been analysed and notified in the EWS, a report is produced including information such as the chemical, physical, pharmacological and toxicological description and other relevant information. This report is useful for the countries to be aware of which substances are being produced and introduced into the illegal market and which of them are being popular in their areas [15–17].

As mentioned above, the use of DOAs is harmful for human health and drug users often experience drug use disorders and moreover they can contract some infections such as HIV or hepatitis C [4]. In Fig. 1, the number of people who took drugs and people with drug use disorders, in the period between 2006 and 2019 is represented. In this figure, it can be observed how the drug consumption varies over the years, showing an increasing trend throughout the years and a stabilisation since 2016. For example, according to the UNODC, 1 out of 18 people over the world between 15 and 64 years took drugs in 2019 (around 275 million people), and of them, almost 13% of people suffered from these disorders, that is almost 1% of the global population [4]. A drug disorder can produce mainly mental disorders that influence on the person's behaviour and brain. Some of the most common disorders are anxiety, depression, bipolarity, attention-deficit hyperactivity disorder (ADHD) or schizophrenia, among others. Moreover, it has been proved that drug users with mild pathologic disorders, may experience worse symptoms over time with the prolongated use of these substances [2].

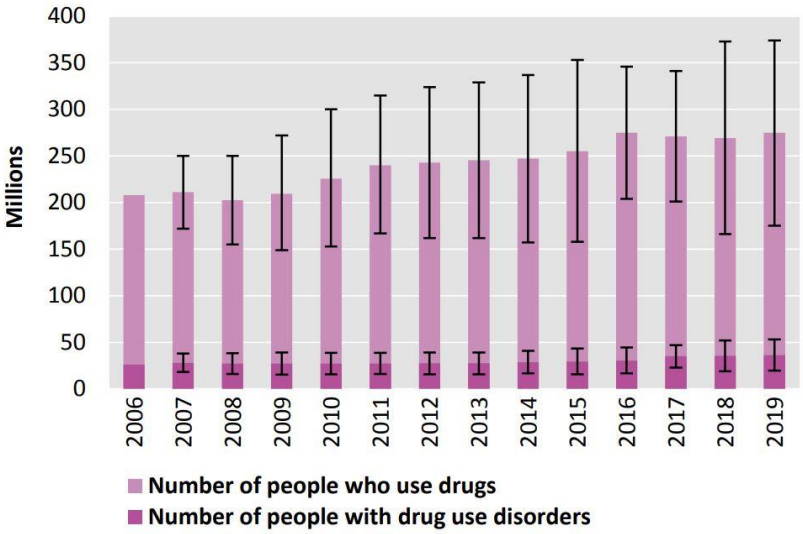

Figure 1. Global number of people who use drugs and people with drug use disorders in the period from 2006 to 2019 [4]

An important feature of some DOAs is that they present a chiral centre in their structures and thus, two enantiomers. Even that both enantiomers have the same chemical structure, they can possess different pharmacological, toxicological and pharmacokinetic activities in the human body. This is because the enantiomers may stereoselectively interact with chiral molecules such as proteins, enzymes and amino acids in the living organism. For chiral drugs, in most cases, only one enantiomer (eutomer) is pharmacologically active and the other (distomer), is not effective or even can be harmful [18]. For that reason, knowing the enantiomeric composition of a substance has gained importance in the forensic and toxicological field. For example, some analgesics can contain an enantiomer of some opioids and without knowing which enantiomer is present in the composition, the analysis would be positive in opioid consumption, even that the analgesic had been taken for medical prescription [18–20].

Another important parameter in the toxicological and forensic field is the metabolism of the substances taken, because when certain biological samples are analysed, not only the parent drug can be found but also their metabolites. Moreover, even that the DOAs metabolism is the main affecting parameter, the concentration of DOAs found in these kind of samples can also vary due to the different metabolic capacities of each individual [21]. Therefore, it is important to know the different substances of abuse available into the market but it is also crucial to understand their metabolism to be able to give a positive result in a drug consumption through the presence of the metabolites even that the parent drugs are not found [22].

There is not a unified classification of DOAs and depending on the information given, they can be classified in different ways. Even though, these classifications are not independent between them and they can be complementary and together, they can provide more information about a substance. This is because they can be based on different characteristics such as their legal status, their effects or the type of substance [3,5]. For example, the DEA classifies DOAs into five different Schedules according to the Controlled Substances Act (CSA) [3]. This distribution is based on the potential abuse, the medical use, the safety and the dependence. In this sense, Schedules I and II are described as the more potential for abuse but differing that the substances in Schedule I are not medically prescribed while the ones in Schedule II are accepted for medical use. Schedule

III is for substances with less potential for abuse than Schedules I and II and medically accepted for some treatments. Then, Schedule IV is designed for drugs with lower potential for abuse than Schedule III with accepted medical use and finally, Schedule V is for those substances with less potential for abuse than Schedule IV and also used for medical treatments. Once the substance is controlled, the DEA propose in the Federal Register to take action either to control, decontrol or reschedule the drug [3,23]. Another classification used by some organisations as DEA or UNODC is by classifying the DOAs according to their different effects in the human body. Thus, they can be distinguished by narcotics, depressants, stimulants, hallucinogens and anabolic steroids. Narcotics are the drugs that wear-out the senses and relieve the pain. Depressants are substances that cause sleep, mitigate anxiety and muscle spasms. Stimulants are considered as the drugs that speed up body's systems. Hallucinogens are substances that change the human perception and character. Finally, anabolic steroids are the compounds that promote muscle growth to improve body's presence [3].

In our case, the classification used in the present Doctoral Thesis is by drug families or substances, which is employed by the EMCDDA in its annual report [5]:

- Cannabis
- Cocaine (COC)
- Amphetamine-type substances (ATS)
- Opioids
- New psychoactive substances (NPS)
- Other DOAs

Next, in the following subsections some more detailed information of the different drug families dealing with aspects such as their prevalence, some of the most important DOAs in the group, their legal status or their metabolization are detailed.

1.1.1. Cannabis

Cannabis is by far the greatest-established drug in the European market and the most consumed in Europe, as it is reported by EMCDDA, which estimates that 1 every 8 young adults between 15 and 34 years have consumed cannabis [24]. There are three species of cannabis plant depending on the place of growth and

their characteristics, *Cannabis sativa* (tropical area), *Cannabis indica* (Central Asia) and *Cannabis ruderalis* (Russian), even that due to the complexity to identify them, *Cannabis sativa* is the generic name. The cannabis plant is composed with more than 450 substances and 66 of them are cannabinoid compounds [25,26]. The term cannabinoid is used for natural compounds as Δ^9-tetrahydrocannabinol (THC), their degradative products such as cannabidiol (CBD) and cannabinol (CBN) or related phytocannabinoids [25,26]. THC is the primary psychoactive compound and it can be found in the dried stem and lower leaves at low percentages (around 1%). The major percentages of THC, which can be 20% or higher, are present in the female flowers and in the resin-producing trichomes that grow among them, which protects the plant from hot and arid climates [24,26]. CBD is psychotropically inactive and has anti-arthritic and antipsychotic properties while CBN is not active nor potentiating compound and has anti-inflammatory and sedative properties. In the case of phytocannabinoids, they can be psychoactive but some of them do not contribute greatly to the effects of cannabis such as THC isomers [26].

Depending on how cannabis is treated and consumed it is known with one name or another. If it is consumed as herb, it is known as marijuana, which is the most prevalent form, while if the resin is extracted from the flower and dried, it is known as hashish. This last has more potency (higher THC percentage) than the herb and is cheaper in the illegal market. As mentioned above, female plants have higher THC percentages and thus, cannabis is usually prepared from them. The main effects of cannabis are euphoria, anxiety, loss of concentration, alteration of time perception, panic attacks and increased appetite, among others. Cannabis use is related with conduct disorders, ADHD, learning disorders, psychosis or violence, among others. [5,24,26,27].

In most European regions, cannabis is legally treated differently than other DOAs since it can be legally cultivated or used for personal consumption and the penalties for its possession or cultivation can be different. In general, the last trends have been to reduce the legal penalties for cannabis users [24]. In the DEA Schedule report, cannabis is in Schedule I, so this means that it has high potential for abuse and it can not be used for medical treatment [3]. However, synthetic versions of THC are legally used for medical prescriptions such as marinol (dronabinol), which is in Schedule III, syndros (dronabinol), which is in Schedule II

or epidoloex (CBD), in Schedule V. In EU, these cannabis-based medicines are also authorised for its medical use in capsules, spray mouth or for vaporising [24]. For example, CBD is known to be used in some pharmaceuticals to treat childhood epilepsy or a cannabis spray based on THC and CBD, which are used in some European countries to alleviate pain and spams [5,24,27–30]. However, the prevalence of cannabis is based on their psychoactive effects and the unpunishing laws related with its consumption. It is the case of the Netherlands, where the practice of tolerance (coffeshops) is also allowed in some municipalities. In Spain, even that the production, supply and public possession of cannabis is banned, the private possession is allowed until a maximum of two plants per household [10,24].

When THC is consumed, not only this compound can be found in the body but also some of its metabolites. As it is shown in Fig. 2, when THC is introduced in the organism, it is mainly hydroxylated to 11-hydroxy-THC (THC-OH), which is an active metabolite. Then, THC-OH is oxidized to the inactive metabolite 11-nor-9-carboxy-Δ9-THC (THC-COOH), which can be then conjugated with the glucuronic acid. Finally, it is excreted as free THC-COOH and glucuronide-bound THC-COOH, which can be hydrolysed to obtain THC-COOH. THC-COOH and its glucuronide are predominant in urine, while THC-OH is in faeces. [27,31].

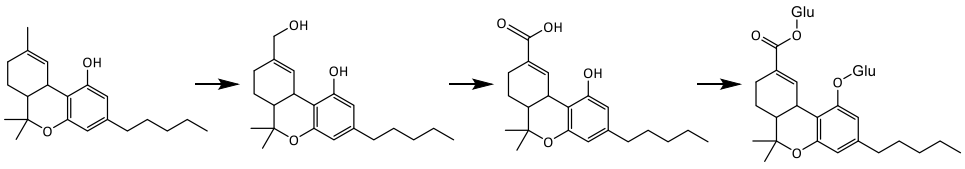

Figure 2. Metabolic route of THC

1.1.2. Cocaine

COC is the most taken illicit stimulant and the second most consumed drug in Europe. It is a natural alkaloid extracted from the *Erythroxylum coca* and *Erythroxylum novogranatense* leaves from Bolivia, Peru and Colombia and it can be taken in two forms: as hydrochloride salt (coke), which is sniffed, snorted or injected and as freebase (crack), which is smoked. However, it is normally

consumed as hydrochloride salt, which is a white powder [4,32]. COC is extracted from the leaves and acidified with hydrochloric acid to obtain the coke. This can not be smoked because it breaks down during pyrolysis. If coke is dissolved in water, mixed with a strong base and then heated, crack is obtained. The name of crack comes from the cracking noise that COC makes when it is smoked [33]. The intensity of the effects produced by COC consumption depends on the rapidity that this substance reaches the brain. In this sense, smoking or injecting COC can reach the brain in seconds causing an intense euphoric feeling known as “rush”. On the other hand, snorting it cause less euphoric sensation and arrive later than smoking or injecting it. More effects of COC consumption are excitation, anxiety, increased heart rate or insomnia, among others [3,32–35].

Even that COC started being produced legally in the 19th century, it is now illegal in most of the regions. In 2008, the INCB called for the suppression of its legal status, and it now appears in the list of Narcotic Drugs Under International Control [36]. According to the DEA, COC is listed in Schedule II as a high potential for abuse substance with accepted medical use in US [3]. In order to reduce the COC supply, an exhaustive control of the main trafficking routes has been experienced in the last years and in this sense, ports and coastal countries play a very important role in COC trafficking routes [37]. For example, Spain has historically been one of the main entry points of COC due to the Colombian and Galician trade, fact that is described in book *Fariña*. Even though, COC routes evolved and apart from Galicia, other cities such as Barcelona, Valencia and Algeciras became entry points of COC in Spain. In the last years, Antwerp (Belgium) has emerged as the principal entry point of COC to Europe and has displaced Spain in this position. Apart from Antwerp, Rotterdam (Netherlands) and Hamburg (Germany) have also become the main entry points of COC due to the large capacities of their port terminals [37,38].

In reference to COC metabolism, as it can be observed in Fig. 3, once COC is taken, it is rapidly metabolised in the liver mainly by hydrolysis to produce its major metabolites, benzoylecgonine (BZE) and ecgonine methyl ester (EME).

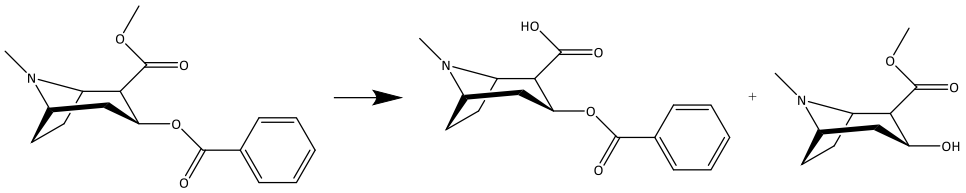

Figure 3. COC metabolism to its major metabolites BZE and EME

Other products metabolised from COC are cocaethylene or norcocaine, which are its minor metabolites [39–42]. The major metabolites BZE and EME remain longer in the body than COC and they are useful to identify COC consumption or also to estimate approximately when it was taken. For that purpose, BZE:COC and EME:COC ratios are usually evaluated in some matrices. Higher COC concentrations compared to their metabolites mean that it is not or only partially metabolised, while lower or no concentrations of COC mean that it is mostly or totally metabolised. Therefore, low ratio values (between 0 and 1), which means higher concentration of the COC than the metabolites, are related to recent drug use and vice versa [43,44].

1.1.3. Amphetamine-type substances

ATS is a group of psychostimulant substances that include the well-known amphetamine (AMP), methamphetamine (MAMP) and 3,4-methylenedioxymethamphetamine (MDMA or ecstasy) among others. Their structures are shown in Fig. 4. Although AMP is more commonly used in Europe than MAMP, the latest is more popular over the world [32,34]. The main effects of ATS are euphoria, increased energy, appetite suppression and hallucinogenic effects in the case of MDMA [45,46].

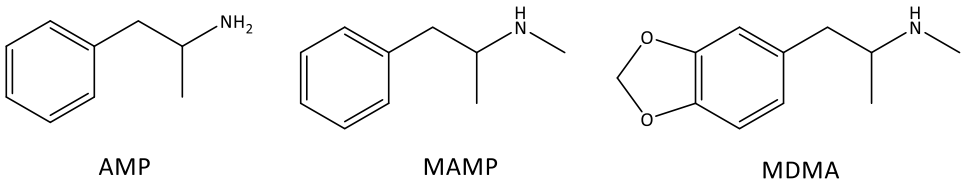

Figure 4. Chemical structures of AMP, MAMP, and MDMA

These substances are chemically synthetised in clandestine laboratories achieving racemic mixtures of R and S enantiomers because the obtention of a single enantiomer is more expensive, even that the potency of the product may be reduced [47]. As it has been previously mentioned, both enantiomers have different pharmacokinetic and pharmacological characteristics. For example, the S-enantiomer of AMP or the prodrug Lisdexamphetamine, is used for the ADHD treatment, while the R enantiomer is responsible of the euphoric, appetite suppression effects, etc. [48–50]. In the case of MAMP, its R-enantiomer is used as a decongestant in some commercial devices while the S-enantiomer is related to stimulant effects [51].

AMP can be found as powder or in pills while MAMP can be found in pills, powder (speed), crystalline (ice) or in base form (paste) [5,46,52]. Regarding MDMA, in 2019 there were 4.6 million seizures of tablets while the seizures of MDMA powder were 2.2 million [5,46]. The crystalline form is usually heated and inhaled or dissolved in water and injected and it is the most potent form of MAMP. In the case of MDMA, it is usually found in tablets but it can also be taken as powder. According to the European Drug Report of 2021 [5], AMP consumers reported that the most used administration route was sniffing (65%), while oral consumption was the second (16%). For MAMP abusers, sniffing was also the most common route (42%) followed by injecting (29%). Most ATS are marked in Schedule II, being highly potential for abuse and accepted for medical use, but only with medical prescription [3]. In this sense, they can be legally prescribed in some cases to treat hyperactivity disorder or narcolepsy [48,53].

Regarding the metabolization of ATS substances in the human body, compounds such as 3,4-methylenedioxyamphetamine (MDA) and 4-hydroxy-3-methoxymethamphetaine (HMMA) for MDMA and p-hydroxymethamphetamine (OHMA) and AMP for MAMP can be found in some biological matrices such as urine [54]. Therefore, the presence of AMP may be due to its consumption or also to the metabolization of MAMP. Moreover, as mentioned before, these compounds can be taken for therapeutic purposes. In this case, AMP or MAMP use can not be differentiated from drug abuse of medical treatment if they are not enantiomerically determined [55].

1.1.4. Opioids

Opioids family refers to the opiates and the analogues synthesized from them, which are substances that mimic the effects of the natural opium, i.e. substances with morphine (MOR)-like properties [29,56,57]. Natural opioids are obtained from the poppy plant (*Papaver somniferum*) such as opium and MOR. There are also semi-synthetic opioids, which are synthetised from natural opium products. Some of these semi-synthetic opioids are codeine (COD), heroin (HER), buprenorphine and oxycodone, among others. For example, as shown in Fig. 5, COD (methylmorphine) is obtained by the addition of a methyl group in the MOR structure and HER is a MOR acetylation product [3,29,57–60].

Opioids can be normally found in the market in different forms such as in pills, powder, skin patches or in liquid form. Depending on the opioid nature, they can be swallowed, sniffed, smoked or injected [3,57]. When these substances are taken, they can cause reduced tension, anxiety, aggressivity or sleepiness, among other effects. However, when they are medically prescribed, the main intention is to produce pain relief [3,59].

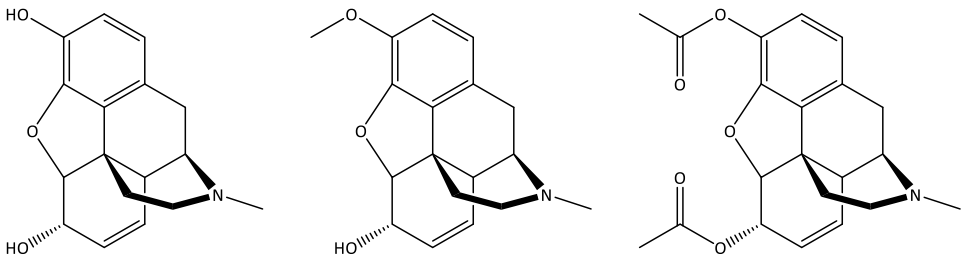

Figure 5. Chemical structures of MOR, COD and HER

The legal status is very different for all the substances of this family as some of them can be used for medical treatments and not all of them have the same effects, abuse potential or dependence. For that reason, their classification varies between Schedule I and Schedule V. For example, HER is included in Schedule I, as it has no medical use, MOR in Schedule II and COD in Schedule III [3]. Another example is buprenorphine, which is in Schedule III and is one of the most used

prescribed medications in EU [61]. Therefore, their control is very exhaustive since they are very popular in the society and moreover, they can be used for medical interventions, for rehabilitations, or for reintegration into the society after a detoxification program.

Opioid metabolism may vary depending on the compound but some of them have the same metabolic route. Opioids that have a hydroxylic group in their structure are conjugated in the liver with glucuronic acid [57]. For example, as it is shown in Fig. 6, HER is rapidly de-acetylated in the body, and it is hydrolysed to 6-acetylmorphine (6-AM), which is then excreted as MOR, hydromorphone in less proportion or as its glucuronides morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) [60,62,63].

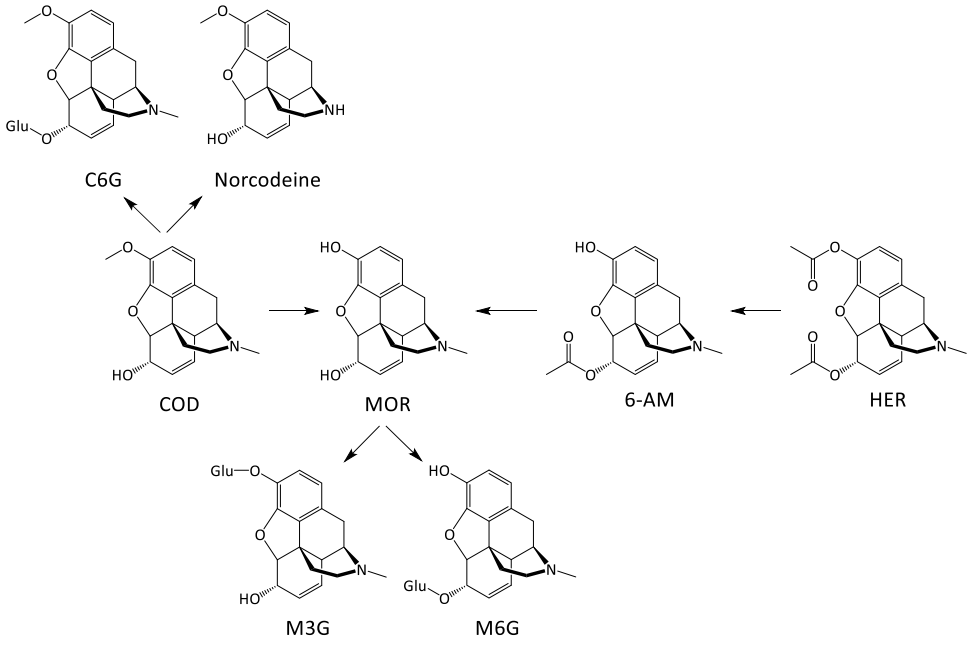

Figure 6. Metabolic route of HER, COD and MOR

In the case of COD, it is also observed in Fig. 6 that it is metabolised to norcodeine, codeine-6-glucuronide (C6G) and to MOR. Then, MOR is also metabolised to M3G and M6G [64]. Therefore, MOR can be found in biological

samples due to its consumption or to the consumption of other opioids such as COD or HER [63–67].

1.1.5. New psychoactive substances

As the drug market is constantly evolving and the drug users searching for new substances is also increasing, new drugs were necessary to satisfy the needs of this demand. Since the 1990s, new compounds named NPS have been introduced into the market. NPS are substances designed to imitate DOAs that are controlled under drug agencies producing similar effects than some of them. Even that they are named as "new", they do not need to be new compounds because it can also be referred to substances synthetised long ago that are nowadays popular [68–70]. They are designed as legal alternatives to other DOAs since they are regularly appearing into the market and their control is very challenging because the process of controlling a substance can take time (in some cases more than a year) and once a substance is controlled, another one is introduced into the market [68,69,71]. For example, in 2019, the Commission on Narcotic Drugs placed 48 NPS under control, which had to be implemented into national laws [72]. Because of their legal status, these compounds are also known as "bath salts", "legal highs" or "research chemicals" and can be bought in some internet websites [73]. In order to improve the control of NPS, the EWS were created to bring faster responses to new drugs. Their process is based on three main steps: initial report; risk assessment and control measures, which can take 6 months for controlling a new substance. Nowadays, more than 1100 NPS have been reported by the UNODC in 133 countries and territories [17,74,75]. Most of NPS are classified in Schedule I according to the CSA as they are highly potential for abuse [3].

There is a diversity of NPS available into the market such as synthetic cannabinoids, synthetic cathinones, synthetic opioids, novel phenethylamines, piperazines, novel benzodiazepines (BZD) and tryptamines, among others [17,34,53]. As it can be observed in Fig. 7, the most important groups of NPS are synthetic cannabinoids, synthetic cathinones and phenethylamines. A total of 830 NPS were being monitored at the end of 2020 and 46 of them were firstly reported in that year. A total of 209 cannabinoids are monitored by EWS, this represents around 25% of all the NPS, while cathinones represents 19% (156) and phenethylamines 12% (102). The 209 synthetic cannabinoids were detected in

Europe since 2008 and regarding the 67 synthetic opioids monitored, they were detected between 2009 and 2020 [17].

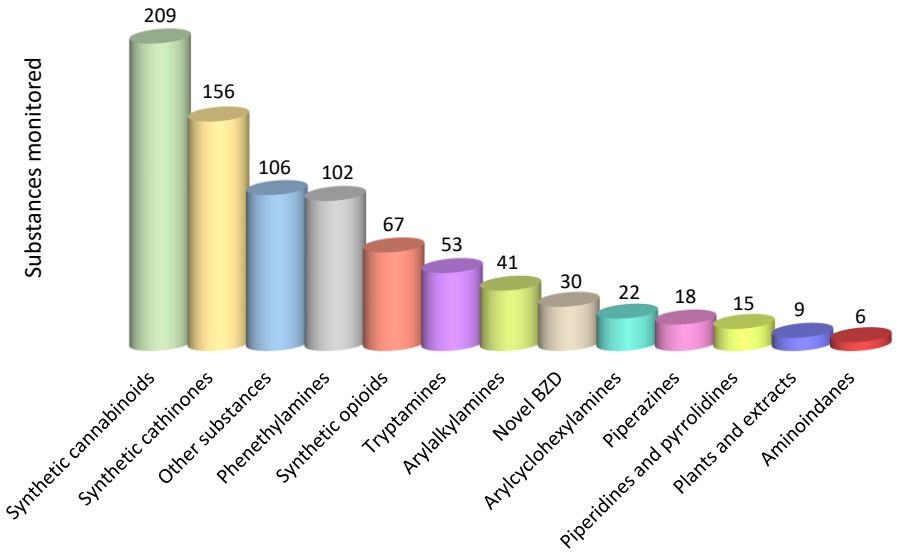

Figure 7. NPS monitored by EWS until the end of 2020 [17]

Most of these groups derive from classic drugs and they are synthesized by minimum structural modifications of these compounds, which produces a very similar but different molecule with similar effects [69]. Among all the NPS, some of the most detected are MDMB-CHMICA (synthetic cannabinoid), mephedrone (synthetic cathinone), carfentanyl (synthetic opioid) and etizolam (novel BZD) [32].

As it has been mentioned, one of the most important groups of NPS are synthetic cannabinoids, which are derived from cannabis and as THC is under international control, these new compounds were created to avoid legislation. They became popular with products as K2 and Spice, which were sold as incenses or smoking mixtures [17,68,76]. Another important NPS family are novel phenethylamines, which are NPS derived from ATS that include ring-substituted AMP-substances. They started being synthetised between 1980 and 1990 by simple variations of natural phenethylamines. They are commercially known as “party pills” and some of the most common phenethylamines are p-

methoxyamphetamine (PMA) and p-methoxymethamphetamine (PMMA) [68,76,77]. Apart from them, there are other groups such as synthetic opioids, tryptamines or novel BZD, among others, that have experienced an increase in their consumption during the last years [17].

Synthetic cathinones stand out as one of the most prevalent NPS in the market after synthetic cannabinoids. In 2019, 60% of drug seizures were due to synthetic cathinones and synthetic cannabinoids, which shows their popularity [5]. As it is shown in Fig. 8, synthetic cathinones (b) are derived from the molecule cathinone (a), found in the *Catha edulis* plant. They are AMP-like alternatives with stimulant effects as their structures are very similar except for the ketone group in the cathinones, as it can be observed comparing Fig. 4 and Fig. 8. As it can be seen in Fig. 8b, synthetic cathinones can present a wide variety of synthetic substances, since different substituents can be placed in four different positions. They can be alkylated or halogenated in the different R^1, R^2, R^3 or R^4 positions to obtain different cathinones. For example, if the cathinone is methylated in positions R^1 and R^4, 4-methylmethcathinone (mephedrone) is obtained, whereas if with a fluor in position R^1 and a methyl in R^4, results in 4-fluoromethcathinone (flephedrone) is obtained. Depending on the substituents, synthetic cathinones can be divided in four groups: N-alkyl compounds or those with an alkyl or halogen substituents in the aromatic ring; methylenedioxy-substituted substances with substituents in the aromatic ring; analogues of natural cathinone with an N-pyrrolidinyl substituent; and methylenedioxyl and N-pyrrolidinyl substituents substances [71,78,79].

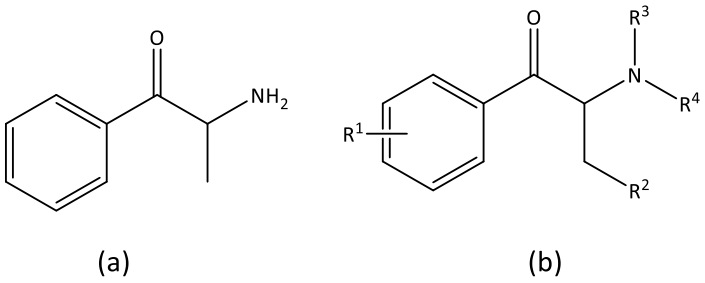

Figure 8. Structure of cathinone (a) and cathinone derivates (b)

Synthetic cathinones are usually sold in white or brown powder for sniffing. As mentioned above, they are known as “bath salts” and when they are sold through different websites, they are market as “not for human consumption”. Their consumption can cause hallucinations, panic attacks, paranoia and anxiety among others [80]. In the same case as ATS, they present a chiral centre and thus, the presence of two enantiomers, which are important to distinguish because of their different metabolic pathways and pharmacokinetic and pharmacological properties in humans. Therefore, in case of being consumed as a mixture, the purity of the substance can be known as well as the enantiomeric percentages [81].

1.1.6. Other drugs of abuse

There can be other DOAs that have not been previously classified in the mentioned families such as ketamine (KET), lysergic acid diethylamide (LSD), BZD or Δ-hydroxybutyrate (GHB) among others [5]. Their structures are shown in Fig. 9, and in particular, diazepam is included to represent the group of BZD as it is one of the most consumed substances in this group. These substances have been poorly monitored by the EMCDDA and due to their lately increasing impact on public health, they are starting to increase their control [5].

KET is a synthetic drug with hallucinogenic effects and it is one of the most popular drugs in the world [3,32]. It is usually sold in a white powder or clear liquid and it can be snorted, smoked or injected. KET may produce state of sedation, relief from pain, amnesia or hallucinations. It is used as anaesthetic for humans and animals and most KET is obtained by stealing veterinary clinics. It is classified in Schedule III due to its medical uses [3]. Once it is taken, it is metabolised in the body to its major metabolite norketamine, which is an active metabolite that has the anaesthetic effects [82].

LSD is a potent hallucinogen derived from the lysergic acid, found in the *Claviceps purpurea*. Even that it was one of the most popular drugs in the 1960s and 1980s, it is now less common. It can be sold in pills, in liquid form or in paper doses, which are placed on the tongue. It is colourless and odourless with a slightly bitter taste and may produce panic reactions and hallucinations. As LSD is not medically prescribed and it has high potential for abuse, it is placed in

Schedule I [3,83]. When LSD is taken, it is extensively metabolised and only 1% is excreted as LSD. Its major metabolite, 2-oxo-3-hydroxy-LSD, is used to detect LSD use in human body [84].

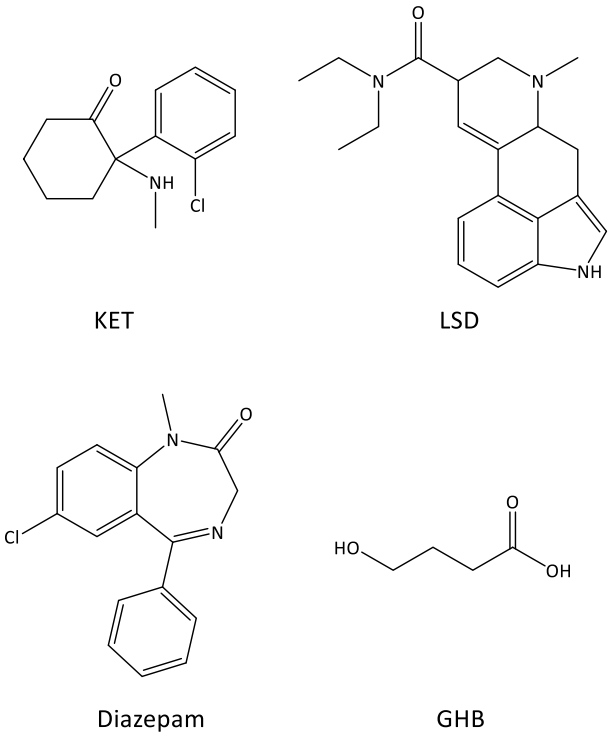

Figure 9. Structures of KET, LSD, diazepam (BZD) and GHB

BZD are the most commonly prescribed sedative drugs. They are controlled substances since they are used in medicine and have a huge variation of potency and doses. However, they have been very popular especially among opioid users in order to potentiate the opioid effect [34,39,53]. They are sold in pills and can cause disinhibition, and strange aggressiveness [85]. Some of the most common are alprazolam, bromazepam or diazepam, which are listed in Schedule IV. Diazepam is the most common prescribed BZD because it rapidly achieved its potency of action and one of the main problems related with this substance is that it is generally used in higher doses than it is prescribed [3,85,86].

GHB is a depressant that produces sedation and anaesthesia and it is very common in sex parties for its disinhibiting action and sedative effect. GHB can also be produced from other substances such as Δ-butyrolactone (GBL) or 1,4-butanediol, which are immediately metabolised to GHB once they are taken [3,53,87,88]. GHB is usually found in pills and in liquid forms and as mentioned, it is usually added to the drinking consumption for sexual assaults. As it is not medically prescribed and marked as highly for abuse, it is classified in Schedule I [3,88]. Its detection is very challenging as the metabolization of GHB is very quickly and only 5% of it is eliminated through urine and moreover, it is difficult to detect after 10 h [88].

1.2. Biological samples for drug testing

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The determination of DOAs in biological matrices is one of the most challenging parts in toxicological and forensic analyses mainly due to the complexity of these matrices that can contain different endogenous and exogenous compounds that may interfere in the analysis. In the last years, the development of analytical methodologies to determine DOAs in biological specimens has been useful to investigate police cases in which a drug abuse is suspected, to monitor patients following a detoxification program or to control the drug demand, among other examples [4,89,90]. Most of the developed methods have been based on the analysis of specimens from drugs abusers following detoxification programs or in post-mortem cases. In some of these studies, different biological samples were analysed to obtain as much information as possible and to detect possible DOAs in their parent form or as metabolites [91–102].

The most commonly used matrices for toxicological and forensic analyses are urine, blood, oral fluid (OF) or saliva and hair. Apart from them, there are other biological specimens such as sweat, breath, bile, meconium and nails among others that have also been of interest [101,103–109]. In Fig. 10, the percentages of the biological matrices employed in research articles focused in the determination of DOAs from 2015 until April 2022 are shown. As it can be observed, urine and blood have been the most frequently analysed matrices representing 84% of the global specimens. OF has been less employed than the previous but it is gaining more importance in the last years as an alternative to blood. Hair has also been used in a similar percentage than OF while meconium is one of the most used matrices in the group of other specimens, even that this group only represents 1% of the global. Every biological matrix comes with a unique set of advantages, drawbacks and challenges and depending on the final purpose required in the analyses, it can be of interest the choice of a particular matrix or a combination of several of them.

It is important to know which information can provide each specimen as once the drug is ingested, and can be detected in OF, it is distributed to different parts of the body via blood. Then, the hair follicles can absorb the drugs from the bloodstream and as most DOAs are lipid soluble, they are absorbed in the tissues and metabolised in the liver prior their elimination through urine. The distribution of DOAs through the human body is different and for that reason, the use of

several specimens can be complementary, because the parent drug may be present in some matrices, whereas in others only the metabolites can be found.

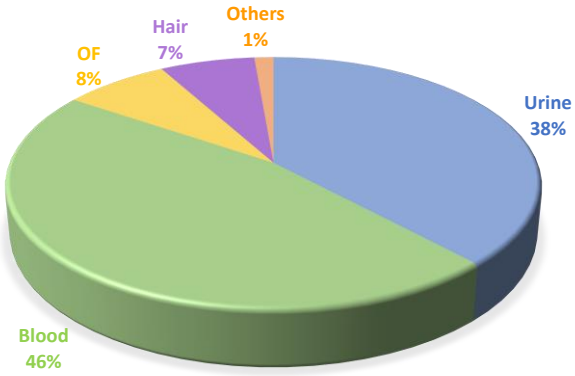

Figure 10. Percentages of the developed methodologies for drug testing in biological matrices in the period between 2015 and April 2022 (Scopus)

The choice of the biological specimen is mainly related to the search of a recent consumption, a consumption in a long time or a monitoring during a detoxification process. In Fig. 11, the detection windows of the most common biological matrices are shown. As it can be observed, in the case of recent drug intake, the most appropiate matrices are blood and OF, while in the case of long-time consumption, the best options are urine and hair. Moreover, urine can be used both for recent and long-term intake, as its detection window is from minutes to weeks. Regarding hair analysis, it can also be used to monitor a patient following a detoxification program. As hair grows at a rate of 1 centimetre per month, each centimetre can be analysed to obtain a continuous tracking of the patient and the last consumption date can also be estimated [101,103,104].

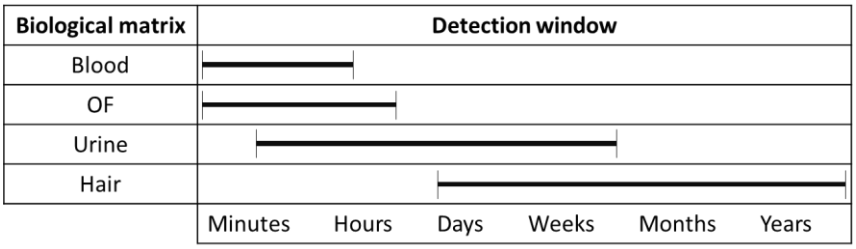

Figure 11. Detection window of the most employed biological matrices in drug analysis

Apart from the different detection windows, the selection of a particular specimen depends on its availability and also on the final purposes of the case, such as monitoring a patient or detecting drug use at the beginning of a detoxification program. Moreover, each biological specimen has particular advantages or drawbacks mainly related with the privacy collection, the need of trained personnel or the sample availability, among others are detailed. In Table 1, the most relevant advantages and disadvantages of the most usually analysed biological matrices are detailed.

Table 1. Advantages and disadvantages of the most employed biological matrices in drug analysis

Biological matrix	Advantages	Disadvantages
Blood	- The concentration found may be related to the amount of drug consumed	- Expensive and invasive collection - Need trained personnel - Health risk such as infection
OF	- Non-invasive sampling - Easy and observable collection - No trained personnel needed - Detection of the parent drug rather than the metabolites	- Limited sample volume - May be contaminated by mouth leftovers
Urine	- High sample availability - Easy and non-invasive - No trained personnel needed - Higher levels of concentrations than blood	- Easy to adulterate if it is not-observable - Presence of metabolites
Hair	- Non-invasive sampling - Easy and observable collection - No trained personnel needed - Retrospective analysis	- Detection after 7 - 10 days - Low concentration levels may not be detected - Hair colour and products may bias the results - It can be removed by shaving the hair

Comparing blood and OF, which are used for recent consumption, the most important differences between these two matrices are based on the collection. While OF sampling is easy and non-invasive and there is no need of trained personnel, blood sampling is invasive, trained personnel is needed, the collection may produce some infections and it is more expensive to collect. However, blood can be used to quantify the amount of drug taken and the availability of sample is higher than in the case of OF, which can also be contaminated for possible food leftovers. In the case of urine, that offers a mid-term window of detection, its collection is easy and non-invasive, it can be obtained in large quantities, no trained personnel are required and usually, higher concentrations of drugs than in blood can be found as it is the main excretion route for all DOAs. Moreover, apart from the parent compounds, their metabolites can also be found, which can be useful to confirm a drug consumption even if the parent drug is not detected. Even though, the main drawback of urine is that if the sampling is non-invasive (not observed), the sample can be adulterated, while if it is observed, it is an invasive collection. Finally, to determine DOAs after several days, weeks or months, hair presents some advantages such as the non-invasive, easy and observable collection. Another advantage is that it can be useful to estimate when a drug was taken (retrospective analysis) by analysing different hair segments. It is also widely used to perform a continuous monitoring of a patient under a detoxification program. The main drawbacks of this matrix are that the drugs can only be detected after 7 or 10 days, that the sample may be contaminated or affected by some hair products and that hair can be shaved and thus, no analysis is possible [100,103,110,111].

The present Doctoral Thesis has been focused on the development of different strategies to determine several types of DOAs in different biological matrices. In particular, urine and OF have been selected because both of them are easy to collect and no trained personal is needed. Moreover, urine is useful for recent drug consumption as well as mid-term consumption. Also, regarding early drug detection, OF has been selected as an alternative sample to blood, and moreover, in the case of its collection is simpler, safer and avoids privacy issues. These two matrices have been the aim of a review included in the next section, in which a comprehensive critical overview of the existing literature focused on the developed strategies for the determination of different DOAs in urine and OF

using chromatographic and related techniques between 2018 and June 2021 have been discussed. This review article has been accepted in the scientific journal *Trends in Analytical Chemistry*, which is attached in Section 1.3.1.

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1.3. Determination of drugs of abuse in urine and oral fluid samples

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DETERMINATION OF DRUGS OF ABUSE IN URINE AND ORAL FLUID SAMPLES
Sergi Pascual Caro

In the present section, the review focused on a critical discussion of the developed methods based on DOAs determination in urine and OF using chromatographic and related techniques between 2018 and June 2021 is presented. This review compiles different research articles focused in that topic and discusses the pre-treatment and analysis techniques used in them. It is divided in two main sections, i.e., urine and OF, and each section is subdivided in sample pre-treatments and analysis techniques. Special attention is paid to novel strategies based on easy, fast and green approaches, as these have been the trends in the last years. Moreover, sensitive methodologies and those that applied them to real specimens from drug users are also highlighted.

As it has been mentioned, the review comprised a period until June 2021, and due to the continuous publication of different analytical methods to determine DOAs in urine and OF, and in order to update the reported methodologies, some of the most relevant articles developed between July 2021 and May 2022 are discussed below. A summary of these methodologies is shown in Table 2 for urine and in Table 3 for OF, in which the analytes, the analysis technique, the sample pre-treatment, the extraction recoveries, the detection and quantification limits (LODs and LOQs) and additional comments are included.

In Table 2, a global overview of the developed methodologies in urine is shown. From this table, it can be observed that after urine collection in a sterile container, a pre-treatment is usually carried out to clean-up or also preconcentrate the analytes and to illustrate the main pre-treatments used in this last period, Fig. 12 shows them with the corresponding percentages. It can be observed that among the 20 reported methodologies, SPE is the most employed technique (59%), including different forms of SPE such as magnetic SPE (MSPE), on-line SPE, dispersive SPE (dSPE) or pipette-tip SPE (PT-SPE). Among these, conventional SPE has been the preferred choice for most of the authors (50%), being mixed-mode cationic exchange sorbents the most used these last months. Apart from SPE, LLE has also been used by several authors (17%), being dispersive liquid-liquid microextraction (DLLME) the used form of this technique in an attempt to reduce the volume of organic solvent to achieve greener strategies. The dilute and shoot strategy has been also employed as an easy and fast procedure, which has been used as the unique pre-treatment in some cases. Finally, other pre-treatments such as QuEChERS (quick, easy, cheap, effective,

rugged and safe) or porous sorptive polymer thin film microextraction (PSP-TFME) have been also used by some authors (12%).

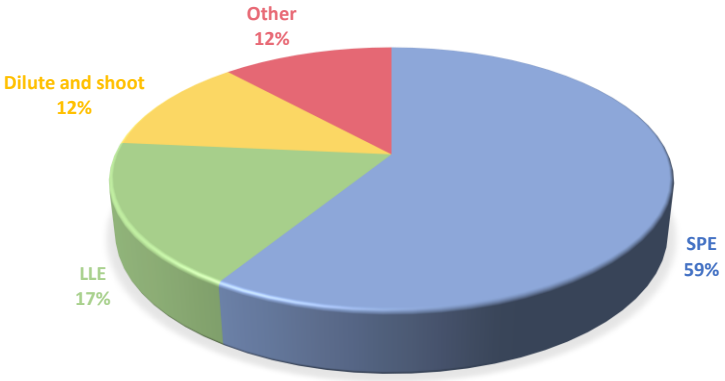

Figure 12. Percentages of the most used urine pre-treatments between July 2021 and May 2022

In general, the published strategies are in line with the last trends since 2018, which are described in the review included in this section and are based on achieving green, fast, multiresidue and simple miniaturised techniques. It is the case, for example, of Mata *et al.* [112], who determined 30 different DOAs using PT-SPE after a hydrolysis step. This strategy allows the extraction of the analytes using very low amounts of sorbent in a pipette tip to obtain easy, green and quick procedures without any specialised instrumentation. They performed the extraction using WAX-S mixed-mode sorbents, which are commercially available. In the case of DLLME, most of the methodologies still use organic solvents such as dichloromethane (DCM), ethyl acetate or chloroform, which are not environmentally-friendly reagents. For that reason, the trend for some authors is to change these solvents and low transition temperature mixtures (LTTMs) have emerged as greener alternatives for their inexpensive, recyclable and non-toxic materials. They present other advantages such as water solubility, ability to dissolve numerous substances, non-flammability, biodegradability and they are very easy to prepare, as they are obtained by simply mixing different starting materials at moderate temperatures [113]. It is the case of Gallo *et al.* [114], who

determined 14 DOAs using DLLME as sample pre-treatment and using choline chloride and sesamol as the starting materials for the LTTM.

After the sample pre-treatment, the analysis has been mostly performed by liquid chromatography (LC) coupled to mass spectrometry (MS) [112,114–125]. In particular, the reported strategies in last months are mainly based on tandem MS (MS/MS), being triple quadrupole (QqQ) the most used detector. Gas chromatography (GC) [126–130] or capillary electrophoresis (CE) [131,132] have also been employed by several authors. However, the main drawback of GC is that as most drugs are not volatile and thus, a derivatisation step is usually needed, which means longer analysis times, while for CE, the sensitivity is in general, lower than LC or GC. Merone *et al.* [123] and Shi *et al.* [124] developed methodologies able to determine 739 and 239 DOAs and their metabolites using LC-QTrap and LC-Orbitrap, respectively. Even that they may not be as sensitive as other methodologies, these are general procedures that allow the determination of a large number of compounds in a single method. Regarding the interest in the enantiodetermination of DOAs, Pascual-Caro *et al.* [133] developed a CE-MS/MS methodology to determine the enantiomers of AMP, MAMP, MDMA and MDA. They evaluated different chiral selectors and BGE compositions to achieve the best separation in the shortest time, achieving their separation in less than 5 minutes in a total analysis time of 22 minutes.

Most of the methodologies from Table 2 are focused on different drug families and ATS and NPS are some of the most studied. Regarding the applicability of these developed methods, some of them have been applied to urine samples from drug abusers. For example, Elboraie *et al.* [130] analysed 21 urine samples by GC-QqQ finding MDMA in 17 of them with concentrations between 456 and 1226 ng mL^{-1}, MAMP in 9 samples between 126 and 271 ng mL^{-1} and AMP in 7 between 136 and 253 ng mL^{-1}. Another example is Ferrari Júnior *et al.* [119], who used LC-QqQ to analyse 16 urine samples and found 22 DOAs in at least one sample: COC (494 – 653 ng mL^{-1}), MDMA (91 – 2730 ng mL^{-1}), eutylone (246 – 415 ng mL^{-1}). Moreover, they found some metabolites such as BZE (59.1 – 13900 ng mL^{-1}) or THC-COOH (165 – 798 ng mL^{-1}), among other DOAs. This shows the wide concentration range at which these substances can be found in urine samples from drug abusers and the importance of having sensitive methodologies able to achieve these concentrations.

For OF specimens, special attention is usually paid to saliva collection compared to urine and in this sense, the main collection routes applied in the last months can be observed in Table 3, which have been by direct spitting or using a collector device. In this last way, there are different commercial brands available into the market and one of the most used has been Salivette®. This device is based on a conical tube containing a solid base (usually a small piece of cotton to absorb the saliva) which is centrifuged to collect the OF. This has been used by some authors for collecting a group of opioids [134] or MDMA or COC [135]. It is important to mention that not all of the commercial devices have the same retention of the compounds. Sobczak *et al.* [136] evaluated 15 different commercially available OF collectors for different compounds. They observed differences in the recovery values up to 100 times greater between different collector devices. For example, AMP achieved substance recoveries of 73, 91, 85 and 94% for different collectors, which shows the variability between devices. Therefore, the use of a particular collector device is crucial to achieve representative results because it can affect the concentration of the analytes found.

Even that the last trends in the determination of DOAs in OF specimens are the same than in previous years, there are some novel methodologies developed by different authors that have been firstly applied in OF for some kind of compounds. It is the case of Soares *et al.* [137], who performed the dried OF spots (DOFS) for a group of 6 antidepressants. This is the first time that this technique is used to determine antidepressants in OF. The DOFS strategy combines the collection and the extraction with the simply use of a paper card in which the saliva spot is left to dry and then extracted with a low volume of organic solvent. Another advantage is the low volume of saliva (1 spot) and of organic solvent used for the analysis. Even that in that application the recoveries were relatively low, with values between 13 and 46%, this is a novel strategy and further research is still needed. Other authors such as Ares-Fuentes *et al.* [134] performed a pre-treatment using fabric phase sorptive extraction (FPSE) for a group of 11 synthetic opioids. They achieved excellent results in terms of recoveries (between 95 and 109%) for fentanyl and other ten derivates. The main advantage of this technique is the simplification of sample preparation by eliminating all the pre-treatment steps that are usually involved in other conventional preparation techniques.

Therefore there is no need of filtration, protein precipitation, centrifugation or solvent evaporation and reconstitution. This means that less organic solvent is used, less analysis time is required and thus, an easier, cheaper, faster and greener sample pre-treatment can be performed [138]. Růžička *et al.* [135] developed a fully automated extraction device coupled to CE that allowed the adaptation of the collector into an extraction design, which was also portable. This was based on a single device containing two syringes for soaking the swab and for extraction, one plastic tube with ACN, eppendorf tubes for collecting the extract and different valves and vacuum pumps. The only manual action was placing the swab in the first syringe. Even that the extraction efficiency was lower than for other strategies, it was very useful and practical.

Even that the literature regarding the determination of DOAs in OF is limited, GC has been widely employed in the last year [134,137,139,140] in comparison to LC [141] and CE [135]. One important application in OF from drug abusers was the one developed by Bergström *et al.* [141], who analysed 18579 routine OF samples from psychiatric patients and addiction clinics by LC-QToF. Results showed that 27% of them were positive in AMP, 25% in buprenorphine, 18% in nordiazepam, 16% in alprazolam and only 1% were NPS [141].

Table 2. Analytical methodologies for the determination of drugs of abuse in urine samples (July 2021 – May 2022)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
16 cathinones	GC-MS	Extraction: MSPE	87 – 99%	LOD: 5 – 10 ng mL^{-1} LOQ: 10 – 20 ng mL^{-1}	Lower experimental costs and solvent usages than LLE and SPE with similar results	[126]
Methadone Tramadol	GC-MS	Extraction: MSPE and DLLME (DCM)	76 – 85%	LOD: 0.032 – 0.073 ng mL^{-1} LOQ: 0.1 – 0.11 ng mL^{-1}	30 samples analysed. 4 in tramadol (2.44 – 241.08 ng mL^{-1}) and 6 in methadone (4.54 – 402.67 ng mL^{-1})	[127]
MOR 6-AM	GC-MS	Extraction: SPE (ISOLUTE HCX) Derivatisation: MSTFA	89%	-	HER confirmation by analysing its metabolites MOR and 6-AM. 78 out of 100 confirmed HER positive	[128]
THC THC-OH THC-COOH CBD	GC-QqQ	Hydrolysis: β-glucuronidase Incubation: 40°C 60 min Extraction: on-line SPE (C18)	77 – 85%	LOD: 0.1 – 1.0 ng mL^{-1} LOQ: 0.2 – 3.0 ng mL^{-1}	Highly automatization and minimal manual labour involved	[129]
AMP MAMP MDMA MDA	GC-QqQ	Extraction: dSPE Derivatisation: MSTFA Incubation: 80 °C 25 min	88 – 95%	LOD: 13 ng mL^{-1} LOQ: 42 ng mL^{-1}	21 samples analysed. 17 in MDMA (456 – 1226 ng mL^{-1}), 15 in MDA (318 – 904 ng mL^{-1}), 9 in MAMP (126 – 271 ng mL^{-1}) and 7 in AMP (136 – 253 ng mL^{-1})	[130]
8 piperazines	LC-QqQ	Hydrolysis: NaOH Precipitation: cold ACN	-	LOD: 0.0042 – 1.419 ng mL^{-1} LOQ: 0.0126 – 4.257 ng mL^{-1}	Simple procedure and short analysis time (less than 7 min)	[115]
GHB, precursors and products	LC-QqQ	Derivatisation: benzoyl chloride	92 – 107%	LOD: 10 – 3000 ng mL^{-1} LOQ: 10 – 5000 ng mL^{-1}	6 samples analysed. GHB (110 – 600 ng mL^{-1})	[116]

Table 2. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
40 DOAs and metabolites	LC-QqQ	Extraction: SPE (ExtraBond SCX)	42 – 93%	LOD: 0.003 – 0.500 ng mL^{-1} LOQ: 0.050 – 1.500 ng mL^{-1}	22 samples analysed. COC (0.48 – 535 ng mL^{-1}), and HER (2.64 – 58.3 ng mL^{-1})	[125]
Diazepam and metabolites	LC-QqQ	Extraction: SPE (Bond Elut C18)	86 – 114%	LOD: 0.01 – 0.5 ng mL^{-1} LOQ: 0.05 – 5.0 ng mL^{-1}	Study of the pharmacokinetics of diazepam and its metabolites	[117]
Mephedrone and metabolites	LC-QqQ	Extraction: SPE (Xtrackt® DAU High Flow)	34 – 98%	LOD: 0.08 – 0.4 ng mL^{-1} LOQ: 0.6 – 1.6 ng mL^{-1}	Intranasal administration. All analytes detected in urine and only 2 metabolites detected at	[118]
14 DOAs	LC-QqQ	Extraction: DLLME (LTTM/ethyl acetate)	55 – 100%	LOQ: 0.01 – 0.37 ng mL^{-1}	Use of low transition temperature mixtures is useful to preserve analytes from photo-degradation	[114]
79 NPS	LC-QqQ	Extraction: QuEChERS	80 – 120%	LOD: 0.1 – 10 ng mL^{-1} LOQ: 0.4 – 16.0 ng mL^{-1}	16 samples analysed. COC (494 – 653 ng mL^{-1}), MDMA (91 – 2730 ng mL^{-1}), eutylone (246 – 415 ng mL^{-1}) and THC-COOH (165 – 798 ng mL^{-1})	[119]
39 DOAs	LC-QqQ	Hydrolysis: β-glucuronidase Incubation: 55°C 30 min Extraction: PT-SPE (WAX-S)	-	LOD: 0.156 – 65.5 ng mL^{-1} LOQ: 0.312 – 125 ng mL^{-1}	Methodology developed for six different samples.	[112]
8 DOAs	LC-QqQ	Extraction: PSP-TFME	85 – 118%	LOQ: 0.05 – 2.50 ng mL^{-1}	Rapid and high-throughput microextraction	[120]

Table 2. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
74 ATS and phenethylamines	LC-QTrap	Dilute and shoot	66 – 125%	LOD: 0.5 ng mL^{-1} LOQ: 1.0 ng mL^{-1}	20 out of 67 samples positive. AMP (2.0 – 4.6 µg mL^{-1}), MAMP (0.7 – 147.1 µg mL^{-1}) and PMA (0.4 – 7.3 µg mL^{-1})	[122]
739 DOAs	LC-Qtrap	Hydrolysis: β-glucuronidase Incubation: 40°C 3 h Dilute and shoot	-	-	High-throughput green method applicable to different matrices	[123]
239 synthetic cannabinoids and metabolites	LC-Orbitrap	Hydrolysis: β-glucuronidase/arylsulfatase Incubation: 55°C 30 min Precipitation: cold ACN	32 – 114%	LOD: 5.0 – 50.0 ng mL^{-1}	107 samples analysed (70 positives). MDMB-4en-PINACA butanoic metabolite was the most detected	[124]
8 DOAs including ATS and opioids	CE-DAD	Precipitation: MeOH and zinc acetate Extraction: DLLME (sodium tetraborate buffer/chloroform)	76 – 91%	LOD: 1.0 – 2.0 ng mL^{-1} LOQ: 3.0 – 6.0 ng mL^{-1}	High sensitivity with short extraction time and low cost	[131]
R/S AMP R/S MAMP R/S MDMA R/S MDA	CE-QqQ	Extraction: SPE (Oasis WCX)	70 – 95%	LOD: 0.6 – 1.5 ng mL^{-1} LOQ: 2.0 – 8.0 ng mL^{-1}	Evaluation of six chiral selectors. Enantiomeric determination of 4 ATS in 21 minutes with CE-MS/MS	[132]

Table 3. Analytical methodologies for the determination of drugs of abuse in OF samples (July 2021 – May 2022)

Analytes	Technique	Sample collection and pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
11 synthetic opioids	GC-MS	Collection: Salivette® Extraction: FPSE	95 – 109%	LOD: 1.0 – 15.0 ng mL^{-1} LOQ: 5.0 – 50.0 ng mL^{-1}	First application of FPSE to the analysis of synthetic opioids by GC-MS	[134]
MDMA and metabolites	GC-MS	Collection: direct spitting Extraction: SPE (SPEC C18)	-	LOQ: 5.0 – 10.0 ng mL^{-1}	Metabolite:MDMA ratios were evaluated but time of last use could not be predicted	[139]
6 DOAs	GC-QqQ	Collection and extraction: DOFS	13 – 46%	LOQ: 10.0 – 100.0 ng mL^{-1}	First application of DOFS to determine antidepressants	[137]
9 DOAs	GC-IMS	Collection: direct spitting Extraction: DLLME (chloroform)	70 – 115%	LOD: 11.0 – 30.0 ng mL^{-1} LOQ: 40.0 – 100.0 ng mL^{-1}	Lower sensitivity than GC-MS but also lower acquisition cost	[140]
71 DOAs	LC-QToF	Collection: Greiner Bio-One Mucus precipitation: freeze-thaw Extraction: SALLE (ACN)	52 – 103%	LOD: 0.25 – 10.0 ng mL^{-1} LOQ: 12.5 – 500 ng mL^{-1}	18579 analysed samples. AMP (27%), buprenorphine (25%), nordiazepam (18%) and alprazolam (16%)	[141]
MDMA, COC and metabolites	CE-UV	Collection: Salivette® Extraction: ACN and centrifugation	18 – 20%	-	Automatization of OF extraction with portable instrumentation	[135]

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1.3.1. Recent chromatographic and related based methods for the determination of drugs of abuse in urine and oral fluid: A review from 2018 to June 2021

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DETERMINATION OF DRUGS OF ABUSE IN URINE AND ORAL FLUID SAMPLES
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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.

Keywords: *Drugs of abuse · Urine analysis · Oral fluid analysis · Chromatographic and related techniques · Toxicological and forensic analyses*

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 Δ9-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].

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 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-methylenedioxy-amphetamine (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-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 micro-extraction 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 minutes, 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, C_{18} 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 metabolized 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 derivatization 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 determining11 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.

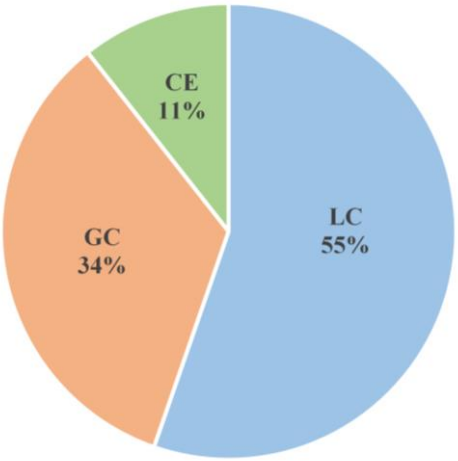

Figure 1. Application of the different chromatographic techniques for drugs of abuse analysis in urine

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 pretreatment 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 countercurrent 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 enantio-determination 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 minutes.

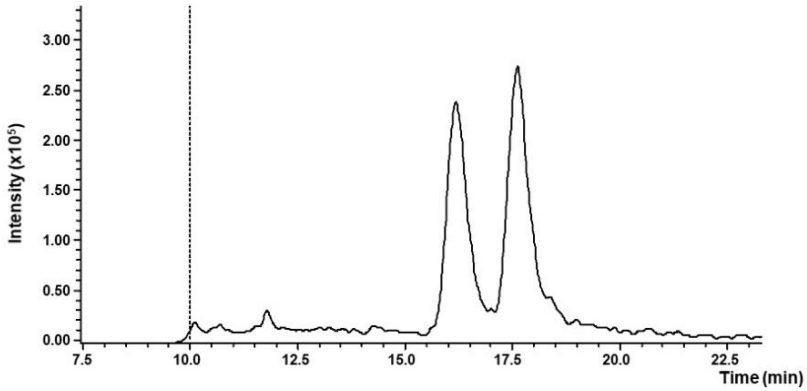

Figure 2. An electropherogram of the in-line SPE-CE method developed by Pérez-Alcaraz et al. for the enantiomeric determination of MDPV in urine. Reproduced with permission from ref. [49]

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 personnel 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

Table 2. 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: α-PVP (67.3 ng mL^{-1}) and OH-α-PVP (560.2 ng mL^{-1})	[65]
CBN CBD THC	GC-MS	Hydrolysis: 1M 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: 0.2M 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 minutes 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]

Table 3. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
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]

Table 4. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
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]
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]

Table 5. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
THC THC-COOH THC-OH	LC-QqQ	Hydrolysis: 10M 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]

Table 6. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
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]

Table 7. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
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]

determining DOAs in OF by chromatographic and related techniques.

3.1. OF sample collection and pretreatment

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].

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 pre-treatment 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 pre-treatment, so the need of a time-consuming and expensive pre-treatment 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

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 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 determining10 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 C18, 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 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. 2). 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 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.

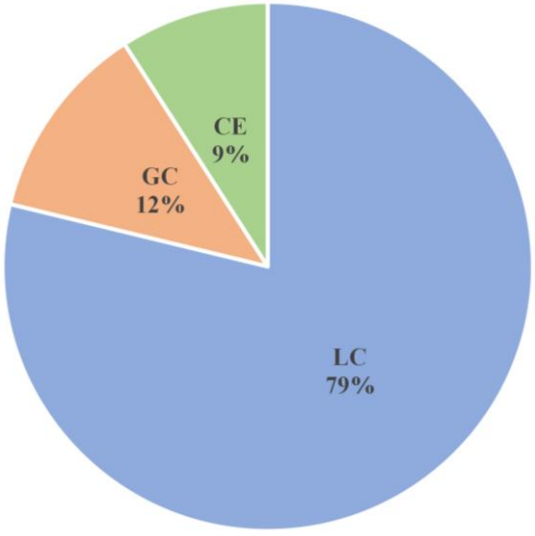

Figure 3. Application of the different chromatographic techniques for drugs of abuse analysis in oral fluid

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 determining12 DOAs in OF from MDMA usersThey 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 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.

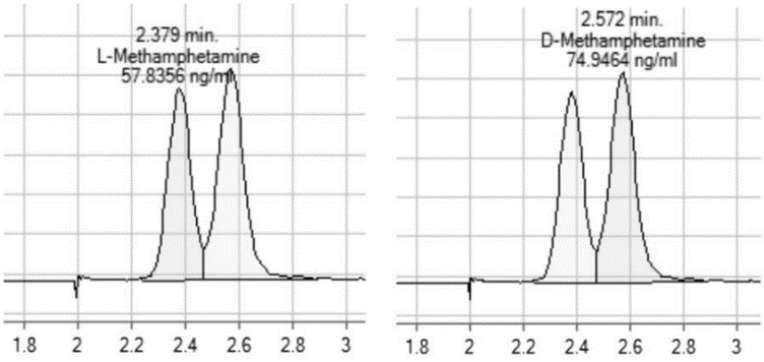

Figure 4. OF analysed with the developed method by Borg et al. using Marfey's reagent for the derivatization of MAMP to produce both enantiomers. Reproduced with permission from ref. [115]

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]

Table 2. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
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]

Table 2. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
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 synthetised 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	LC-QqQ and LC-HRMS	Collection and extraction: DOFS	-	-	229 samples analysed. NPS detected in 17 of them	[129]
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]

Table 2. (Continued)

Analytes	Technique	Sample pre-treatment	Recovery	LOD and LOQ	Comments	Ref.
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 analogs	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]

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CHAPTER 2. OBJECTIVES

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The main objective of the present Doctoral Thesis is the development of different analytical methodologies able to determine several DOAs in urine and OF samples. Moreover, high sensitivity is aimed to achieve the usual levels at which these compounds are present in biological samples from drug abusers (from low ng mL^{-1} to high levels of ng mL^{-1}). For this, different sample pre-treatments have been evaluated and instrumentation such as LC and CE is coupled to MS detection to achieve the mentioned concentration levels.

To the end of achieving this main purpose, different specific objectives have been stablished:

- Evaluate the efficiency of a collector device for the sampling and pre-treatment of OF to determine a group of synthetic cathinones.
- Compare different MS detectors such as MS/MS and HRMS for the determination of synthetic cathinones in urine and OF.
- Test different chiral selectors for the enantioseparation of different ATS and metabolites in urine by CE-MS/MS.
- Study and compare different mixed-mode commercial extraction sorbents for the pre-treatment of urine by SPE to determine different groups of DOAs and some of their metabolites.
- Evaluate a novel microextraction technique to achieve a more environmentally-friendly strategy in a method focused on the determination of a large group of DOAs in urine samples.
- Apply the developed methods to urine samples from drug users to demonstrate their suitability for forensic and toxicological analyses.

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CHAPTER 3. EXPERIMENTAL PART, RESULTS AND DISCUSSION

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As it has been previously mentioned in the Introduction section, DOAs are continuously evolving and new synthetic derivates are emerging into the illegal market. In this market different families can be found as cannabis, COC, ATS, opioids, NPS and other DOAs. Even that new synthetic drugs are continuously appearing, the most consumed drugs are from classic families such as COC, ATS, BZD and some opioids. In this sense, there is an unceasing research focused on the development of new methods able to determine these new and classical drugs in biological matrices. Even that there are numerous methods for that purpose, there is a need on developing easy, green and sensitive methods for determining these compounds at low levels of concentration (ng mL^{-1}). For that reason, this Doctoral Thesis is focused on the development of sensitive methods able to determine both classic and synthetic drugs in biological matrices and more precisely, in urine and OF.

The experimental part has been developed in the research group "Cromatografia, Aplicacions Mediambientals (CROMA)" from Universitat Rovira i Virgili, in Tarragona. This group has a wide experience in the determination of DOAs in environmental and biological matrices such as river water samples, urine or hair. Moreover, some of the previous Doctoral Thesis of the group have been focused on the development of analytical methods to enantiomerically determine DOAs using LC and CE. The studies reported in this Doctoral Thesis were financially supported by the Ministerio de Ciencia, Innovación y Universidades (CTQ2014-52617), European Regional Development Fund (ERDF) (CTQ2017-88548-P and PID2020-114587GB-I00) and the Iniciativa d'Ocupació Juvenil (PEJ2018-003102-A).

This chapter includes the experimental part of the research and the results and the discussion of the main findings obtained through the different studies carried out during the present Doctoral Thesis. They are divided in three different sections: the first focused on the determination of synthetic cathinones in urine and OF (section 3.1), the second on the determination of amphetamine-type substances in urine (section 3.2) and the third related to the determination of several drugs from different families in urine (section 3.3). All these sections include a brief introduction to summarise the background of the research and a discussion of the most relevant obtained results. All the papers derived from this Doctoral Thesis have been published or are in process of publication in different

international scientific journals and they are presented herein in scientific article format. A list of all these papers has been included in Appendix II.

3.1. Determination of synthetic cathinones in urine and oral fluid

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Among all DOAs, synthetic cathinones stand out as one of the most consumed NPS over the world because of their similarities with other classic drugs such as ATS. Moreover, they are one of the most challenging substances to control because every year different new cathinones are introduced onto the market. Some of the most consumed cathinones are 4-methylmethcathinone (mephedrone), (3,4-methylenedioxy-N-methylcathinone (methylone) and 3,4-methylenedioxypyrovalerone (MDPV) [1–5].

As it has been previously mentioned in the Introduction section, different authors have focused on the determination of synthetic cathinones in urine and OF. For this purpose, LC, GC and CE have been employed after a pre-treatment step such as SPE, LLE or dilute and shoot strategy. These last developed methodologies aimed to obtain easy, fast and sensitive strategies to determine these substances in biological samples. In that case, the instrumentation used and the possible preconcentration techniques play an important role if low concentration values are required.

Following with the investigation of our research group related with these drugs, which has been focused on the determination of synthetic cathinones in environmental water matrices and the enantiodetermination of these compounds by CE and LC, the present section is based on the determination of several synthetic cathinones in two biological samples such as urine and OF by LC [6–11]. This last matrix has emerged in the last years as an alternative to blood for its easiest and non-invasive collection and gives information about recent consumption while urine is one of the most employed specimens to obtain information about mid-term drug consumption.

In the first study, a method for the determination of eleven synthetic cathinones in urine was performed by SPE followed by LC coupled to HRMS using Orbitrap as analyser. Two different cation-exchange sorbents (Oasis WCX and Oasis MCX) were evaluated to clean and extract the analytes from urine. This selection was made according to previous experience in our group working with these two sorbents for the determination of synthetic cathinones in environmental water samples [7]. The influence of different parameters affecting the SPE procedure were tested and optimized to get high preconcentration

factors. With the use of the SPE and the LC-HRMS, low levels of ng mL^{-1} could be achieved.

The second study was based on the development of a method to determine the same group of synthetic cathinones as in the first study but in this case, in OF and using LC coupled to MS/MS with QqQ as analyser. OF can be collected in different ways but the most employed is using a swab to adsorb the saliva of the mouth and Salivette® was chosen for this research. The last trends have been the simplification of the procedure and the reduction of analysis times [12–15]. In this sense, the main objective of this study was to perform an easy sample pre-treatment in the same collector device to obtain a fast, easy, green and sensitive methodology.

With the objective of comparing HRMS and MS/MS, the two analysis techniques used in the previous studies (LC-HRMS and LC-MS/MS) were used to evaluate their advantages and disadvantages to determine synthetic cathinones in urine and OF samples. For this purpose, the methodology developed by LC-HRMS was also validated with the LC-MS/MS and vice-versa. The comparison between LC-MS/MS and LC-HRMS was focused on three main aspects: method recoveries (R_{app}) and matrix effects (ME); linearity, MDLs and MQLs; and accuracy.

In sections 3.1.1., 3.1.2. and 3.1.3. the three developed studies focused on synthetic cathinones are presented in scientific article format. These articles have been published in the following scientific journals: Forensic Toxicology (2020) 38:185-194; Journal of Analytical Toxicology, 2021;45:693–700; and Separations 2020, 7, 53.

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3.1.1. Solid-phase extraction based on cation-exchange sorbents followed by liquid chromatography-high resolution mass spectrometry to determine synthetic cathinones in urine

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Abstract

Purpose Cathinone derivates have become very popular as legal highs and are easily obtained on the market as "bath salts", getting around the legislation due to their modified structure based on banned substances. The aim of this project is to evaluate two mixed mode cation-exchange sorbents in solid-phase extraction (SPE) and their efficacy in pretreating urine samples for the determination of synthetic cathinones by liquid chromatography-high resolution mass spectrometry (LC-HRMS).

Methods A method for determining a group of eleven trending synthetic cathinones in urine using SPE followed by LC-HRMS using Orbitrap as analyser was developed and validated.

Results In the extraction step, two different cation-exchange sorbents were evaluated and compared, a weak one (Oasis WCX) and a strong one (Oasis MCX). Better results were obtained for Oasis MCX in terms of recoveries and matrix effects (lower if a clean-up step was applied). Method quantification limits were set at 0.2 ng mL^{-1} and method detection limits were between 0.04 ng mL^{-1} and 0.16 ng mL^{-1}.

Conclusions The results obtained enabled the studied compounds to be quantified at the usual levels at which they are present in urine samples. Moreover, these limits were lower than the ones found in the literature using similar extraction strategies, which shows that the reported strategy can be a useful tool in forensic and toxicological analysis.

Keywords: *Synthetic cathinones · Solid-phase extraction · Cation-exchange sorbents · Liquid chromatography high-resolution mass spectrometry · Urine samples*

Introduction

The number of people who take drugs is increasing every year since some of the new substances that appear on the market are not prohibited [1]. Nowadays, new psychoactive substances (NPS) are the trending drugs. They are a type of synthetic drugs that include synthetic cathinones and cannabinoids, among others. From 2009 to 2016, 106 countries and territories have reported the emergence of 739 different NPS [2]. NPS can be found under different names, for instance: "legal highs", "bath salts" and "research chemicals". They can be bought easily and legally, because they are modified compounds which are not prohibited by law [3]. The reason they are not banned is because the process of updating the law can take more than a year due to all the requirements and by the time one substance is banned, another one is introduced into the market [4].

This project is focused on synthetic cathinones, which represent a significant portion of the NPS market. They are stimulants obtained from the substance cathinone and they can be found in the khat plant [5]. They are β-keto phenethylamines and structurally they are very similar to amphetamine or methamphetamine. The effects of cathinone consumption are similar to the effects produced by other drugs; for example, paranoia, hallucinations, increased friendliness, increased sex drive, panic attacks, and excited delirium [5, 6]. These compounds have similar effects to other more expensive drugs and are easily obtained on the internet.

When synthetic cathinones are taken, they are eliminated through the urine and they can reach superficial waters. Thus, the presence of cathinones in environmental and biological samples has been widely studied [7–16]. In the case of biological samples, urine is commonly used to determine the presence of drugs, because it is easy to sample and also due to the mid-window of detection [17]. Cathinones are absorbed in the tissues and due to their lipidic solubility, they are present in the liver prior to their elimination through the urine, where, in some cases, they have been found at low levels of ng mL^{-1} 24 h after consumption [18]. Apart from urine, there are other biological samples, such as saliva, breath, blood, sweat and hair, that have also been studied [16, 19–23]. In this study, we have focused on the parent compounds of the synthetic cathinones since in previous studies they were found in environmental water samples, so they probably are not or are only partially metabolised [11, 15].

Nowadays, the analysis of this type of drug is a subject of interest for several authors [8–13, 20, 21, 24, 25]. Synthetic cathinones can be determined either by liquid chromatography (LC), gas

chromatography (GC) or capillary electrophoresis (CE). GC is used in forensic and clinical analyses in some laboratories [7, 26]. However, the main drawback of this technique is that most cathinones need a derivatisation prior to being analysed and they can decompose due to the high temperatures usually used [26]. The use of CE is interesting from the point of view of chiral separation, since the enantioseparation can be achieved by simply adding a chiral selector to the background electrolyte [19, 27, 28]. How- ever, the most prevalent technique used for the separation of cathinones is LC, because it overcomes the disadvantages of the other techniques [9–13, 20, 21, 29]. In addition, most of the studies focused on determining cathinones in urine, use a mass spectrometry (MS) detector, and more precisely, tandem MS detector using triple quadrupole (QqQ) [14, 30]. However, the literature in which the detection is performed with high-resolution mass spectrometry (HRMS) is limited to a few articles using quadrupole (Q)-Orbitrap [9, 10] and Q-time-of-flight (QToF) [12, 13]. This present research is focused on using Orbitrap to contribute to increasing the studies in this area.

For urine samples, SPE is the most commonly used technique for extracting the analytes from the sample matrix, as it achieves high recoveries due to the different sorbents available [9, 10, 13, 14, 20, 21, 31–33]. One type of sorbent that combines capacity and selectivity is mixed-mode ion-exchange sorbents. In the literature, some authors have evaluated these types of solvents to extract different compounds from urine samples [9, 10, 13, 32, 33]. In the determination of the opioid dermorphin by Guan et al. [33], they used Oasis MCX cartridge for the SPE. The authors obtained excellent extraction effi-ciencies in urine (between 86 and 92%) and excellent intra-day and inter-day accuracies (between 91 and 100%). To determine tricyclic antidepressants, Chambers et al. [32] used an Oasis WCX as the extraction cartridge. The authors achieved recoveries from 92 to 104% and matrix effects lower than 15%.

The objective of the present study is to develop and validate a method for determining synthetic cathinones in urine by SPE followed by LC-HRMS using Orbitrap as the analyser. Based on the mentioned literature and considering the excellent recoveries reported with Oasis WCX and Oasis MCX for other drugs in urine, the present study tests and compares these two cartridges for the first time for the extraction of cathinones from urine. Therefore, it is important to evaluate these two sorbents in terms of their recoveries and matrix effects to obtain low detection limits. This application has not been reported before using Orbitrap, which might achieve the required concentration levels of the target

compounds in urine and allows the retrospective analysis. It is also more affordable than other HRMS detectors.

Materials and methods

Standards and materials

The cathinone standards were purchased from LGC Standards (Luckenwalde, Germany). The cathinones chosen for this study were: 4-fluoromethcathinone (flephedrone), N-Ethylcathinone (ethcathinone), buphedrone, 2-methylmethcathinone (2-MMC), butylone, 4-methylmethcathinone (mephedrone), 4-methylethcathinone (4-MEC), beta-ethylmethcathinone (pentedrone), 3,4-dimethylmethylcathinone (3,4-DMMC), alpha-pyrrolidinovalerophenone (alpha-PVP) and methylenedioxypyrovalerone (MDPV).

Individual stock solutions of these analytes were prepared at 100 mg L^{-1}, 1000 mg L^{-1} and 2000 mg L^{-1} depending on the compound, using methanol (MeOH) as solvent and they were kept in the freezer at −20 °C. A working mixture solution containing all the analytes at a concentration of 1 mg L^{-1} was prepared in MeOH and the diluted standard solutions were further prepared in H_2O. They were also kept in the freezer at −20 °C.

Ultra-gradient HPLC grade MeOH, acetone and water for LC–MS were purchased from J.T. Baker (Deventer, The Netherlands). Acetonitrile (ACN) for LC–MS was obtained from Chem-Lab (Zedelgem, Belgium). Formic acid (HCOOH), ammonium hydroxide (NH_4OH), hydrochloric acid (HCl), sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Ultrapure water was obtained using a water purification system (Veolia, Sant Cugat del Vallès, Spain).

Oasis WCX (weak cation-exchange) and Oasis MCX (strong cation-exchange) (150 mg/6 mL) extraction cartridges for

the SPE process were purchased from Waters Corp. (Milford, MA, United States).

Instrumentation

The method optimization was performed using a Hewlett Packard 1100 HPLC system with a diode array detection (DAD) from Agilent Technologies (Waldbronn, Germany) controlled by Agilent Chemstation software. The column used was a Luna Omega 5 µm Polar C_{18} (150 mm x 4.6 mm, 5 µm) from Phenomenex (Torrance, CA, United States) with a Security Guard from Phenomenex.

The method validation and the sample analysis were performed using a Thermo Scientific Accela 1250 UHPLC system (Bremen, Germany) equipped

with an Accela Autosampler automatic injector and an Accela 1250 pump coupled with a Thermo Scientific Exactive Orbitrap™ mass spectrometer. The HRMS instrument is also equipped with a heated electrospray ionization (HESI) source and a higher-energy collisional dissociation (HCD) cell to fragment the analytes.

Chromatographic conditions

For the LC-DAD, the temperature of the column was set at 35 °C. The mobile phase flow rate was set at 0.6 mL min^{-1} and the sample volume injection was 20 µL. The mobile phase consisted of A: 0.1% HCOOH in ultrapure water and B: 0.1% HCOOH in ACN in gradient mode. The gradient started at 15% B, which was held for 5 min and increased to 35% in 5 min, then increased to 80% in 4 min and to 100% in 1 min and held for 2 min before returning to the initial conditions in 1 min and maintained for 4 min. All the com- pounds were detected at 260 ± 10 nm except for butylone and MDPV, which were detected at 325 ± 10 nm.

In the case of the LC-HRMS, the mobile phase composition, flow rate, gradient elution and column temperature were the same as for LC-DAD. For the HRMS, the gas flow rates and temperature parameters were optimised to obtain the highest response for all the analytes with positive ionisation and the following optimised parameters were used: sheath gas, 60 AU; auxiliary gas, 5 AU; capillary voltage, 30 V; spray voltage, 2 kV; tube lens voltage, 80 V; skimmer voltage, 24 V. The heater and capillary temperatures were set at 400 °C and 350 °C, respectively. The probe position adjustment was side to side 0, vertical B and micrometre 1.

For data acquisition, two time windows were used in positive mode (0–11.75 min and 11.75–15 min) with two alternating scan events in each window: full scan at 50,000 FWHM with 250 ms of injection time for the first one and fragmentation scan at 10,000 FWHM with 50 ms of injection time for the second one. The optimum collision voltages were 15 eV for the first window and 25 eV for the second one. The optimisation was performed in full scan at high resolution in a mass range between 60 *m/z* and 300 *m/z*.

Urine collection and preparation

The pooled urine samples were obtained from non-addicted volunteers, mixing the urine of different individuals, so we included urine from women and men of different ages. They were collected in polypropylene tubes and kept in the freezer at − 20 °C. Before their analysis, 5 mL of a mixture of urine and phosphate buffer solution adjusted to pH 6 (0.15 M NaH_2PO_4 and 0.05 M Na_2HPO_4) at a ratio of 50:50 (v/v), were loaded in a 150 mg Oasis MCX cartridge after its activation with 5 mL of MeOH

and its conditioning with 5 mL of phosphate buffer solution (pH 6). After the sample loading, the cartridge was washed with 2 mL of MeOH, and finally, the analytes were eluted with 2 mL of 5% NH_4OH in MeOH. Then, 100 µL of 1% HCl in MeOH were added to the methanolic solution. The extracts were finally evaporated to dryness under a gentle stream of N_2, reconstituted with 1 mL of mobile phase at initial conditions, filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter, and then transferred to a vial for their analysis.

Validation

For the instrumental validation (LC-HRMS), the linearity, which was studied at 8 different concentrations (0.1, 0.5, 1, 10, 25, 50, 100 and 250 ng mL^{-1}), detection limits and quantification limits (IDLs and IQLs) were evaluated. Due to the minimal noise when Orbitrap is used, IDLs were considered as the lowest detectable concentration with a signal intensity higher than 1 x 10^3 for the protonated molecule and for the fragments, while IQLs were defined as the lowest point of the calibration curve.

In the case of the method validation, the linearity, repeatability (intra-day), reproducibility (inter-day), method detection limits and method quantification limits (MDLs and MQLs), extraction recovery (R_{SPE}), matrix effect (ME) and apparent recovery (R_{app}) were studied [34]. The retention time, the ion ratio and the error mass were considered to confirm the presence of the compound [35]. The retention time had to correspond to a tolerance of ±2.5 %, the ion ratio ±20 % and the error mass to a maximum of 5 ppm.

The linearity of the method based on a matrix-matched calibration curve was studied for each compound by evaluating the coefficient of determination (r^2) of urine samples spiked prior to the SPE process with cathinones at different concentrations: 0.05, 0.1, 0.2, 0.4, 1, 4, 20, 40, 60, 100, 150 and 200 ng mL^{-1}. The repeatability and reproducibility values, in terms of relative standard deviation (% RSD), were obtained by analysing five replicates of urine samples spiked at 40 ng mL^{-1} prior to the SPE on the same day and on five different days, respectively. MDLs corresponded to a concentration of the cathinones in urine whose signal intensity was higher than 1 x 10^3 for the protonated molecule and for the fragments. MQLs were defined as the lowest point in the matrix-matched calibration curve.

The % R_{SPE} was considered to be the recoveries obtained in the SPE procedure alone and were calculated as the ratio between the concentration obtained when a urine sample was spiked before the SPE procedure and that obtained from direct injection of the standard in the LC-DAD.

The ME was calculated using the following expression:

$$\%ME=[(C_{spiked}/C_{STD})x100]-100$$

(Equation 1)

Where C_{STD} is the concentration of the standard injected and C_{spiked} is the concentration obtained from the urine extract spiked at the same concentration as C_{STD} after the SPE, both injected in the LC-HRMS. A negative value of % ME means a signal suppression and a positive value is related to a signal enhancement.

The % R_{app} was determined as the recovery of the entire method and it was calculated from the ratio between the concentration obtained when a urine sample was spiked before the extraction procedure and that obtained from direct injection of the standard in the LC-HRMS.

Results and discussion

Separation and detection

The Luna Omega 5 µm Polar C_{18} column (150 mm x 4.6 mm i.d., 5 µm) from Phenomenex with a precolumn was chosen to separate the cathinones because of its selectivity and good retention for polar analytes. The composition of the mobile phase was A: 0.1% HCOOH in ultrapure water and B: 0.1% HCOOH in ACN, which was selected based on the literature [9, 11]. ACN and MeOH were studied as mobile phase B solvents, obtaining a better separation in the case of ACN.

First, the DAD detector was used for the experiments to evaluate the two SPE sorbents. The cathinones were identified by individual injections and the optimal wavelength of each compound was studied to achieve the maximum response. The optimal wavelength for most compounds was 260 nm; however, for butylone and MDPV it was 325 nm, and hence, the data acquisition was performed at both wavelengths. It is important to mention that all the compounds were chromatographically separated except for flephedrone and ethcathinone, which had the same retention time and the same optimal wavelength. Although different gradients were tested, it was not possible to separate them without considerably increasing the analysis time. Therefore, with LC-DAD it was only possible to include one of them. Ethcathinone was chosen due to its prevalence in drug users in comparison with flephedrone [5]. Nevertheless, both of them were determined using LC-HRMS because of their different protonated molecules, as shown in the following section.

As already mentioned in the introduction section, the main aim of this study was to develop a method able to determine low concentration levels

of synthetic cathinones in urine as these drugs are usually at low levels of ng mL^{-1} 24 hours after consumption [18]. Thus, in order to achieve this objective, a determination with LC-HRMS using Orbitrap as analyser was applied. The first step consisted in the optimization of the different parameters that can affect the ionization and the transfer to the Orbitrap analyser. The transfer parameters optimised were capillary voltage (25-60 V), tube lens (60-120 V), skimmer (15-40 V) and capillary temperature (250-350 °C). The structure of cathinones allows them to be ionised preferably in positive mode. The optimised ionization parameters were sheath gas (10-60 AU), auxiliary gas (2-10 AU), spray voltage (2-5 kV), heater temperature (300-400 °C) and probe position adjustment side to side (-1 to +1), vertical (A, B, C or D) and micrometre (0-2). In addition, the HCD energy was optimised (10-60 eV) in order to observe two fragment ions for each compound, as shown in Table 1 and in agreement with other studies [9, 11, 36]. The optimum parameters are detailed in the experimental part. The fragmentation parameter was optimised by direct injection of individual standards at 1 mg L^{-1} in H_2O:MeOH (85:15, *v/v*) along with the mobile phase at 65% A and 35% B. All the cathinones had an optimal collision energy of 15 eV, except alpha-PVP and MDPV for which it was 25 eV. The difference in the collision energies can be explained because of the presence of pyrrolidinyl substituents in alpha-PVP and MDPV, which are not present in the other compounds.

Table 1. Retention time, protonated molecule, the two fragment ions and the ion ratios of the studied cathinones.

		Protonated molecule $[M+H]^+$		Fragment 1			Fragment 2		
Analyte	**Rt (min)**	**Formula**	**Accurate mass *m/z***	**Formula**	**Accurate mass m/z**	**Ratio $\frac{[M+H]^+}{F1}$**	**Formula**	**Accurate mass *m/z***	**Ratio $\frac{[M+H]^+}{F2}$**
Flephedrone	6.01	$C_{10}H_{13}FNO$	182.09757	$C_{10}H_{11}FN$	164.08755	38	C_9H_8FN	149.06408	10
Ethcathinone	6.12	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	23	$C_9H_{10}N$	132.08132	12
Buphedrone	7.46	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	19	C_9H_9N	131.07350	6
2-MMC	8.72	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	30	$C_{10}H_{11}N$	145.08915	16
Butylone	8.77	$C_{12}H_{16}NO_3$	222.11247	$C_{11}H_{12}NO$	174.09189	14	$C_{12}H_{14}NO_2$	204.10245	17
Mephedrone	9.20	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	34	$C_{10}H_{11}N$	145.08915	14
4-MEC	10.47	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	30	$C_{10}H_{11}N$	145.08915	9
Pentedrone	10.90	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	16	$C_9H_{10}N$	132.08132	10
3,4-DMMC	11.60	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	37	$C_{11}H_{13}N$	159.10480	12
Alpha-PVP	12.11	$C_{15}H_{22}NO$	232.16959	C_7H_7	91.05478	15	C_7H_5O	105.03404	5
MDPV	12.48	$C_{16}H_{22}NO_3$	276.15942	$C_8H_{16}N$	126.12827	8	$C_8H_7O_2$	135.04461	9

The chromatographic conditions optimised for LC-DAD were the same used in LC-HRMS. Even that some compounds are not separated by their retention time, with the use of HRMS, they can be discriminated according to their protonated molecule and fragments (Table 1).

The ILDs and IQLs were evaluated under the optimal conditions found for LC-HRMS. ILDs ranged from 0.10 ng mL^{-1} to 0.40 ng mL^{-1} and the IQLs were 0.5 ng mL^{-1} for all the cathinones under study. Moreover, good linearity between the concentration points of IQL and 250 ng mL^{-1} with r^2 higher than 0.999 was obtained with standards.

Solid-phase extraction procedure optimization

In the analysis of urine, SPE is one of the techniques most frequently used to extract and preconcentrate the cathinones [20, 21]. Cathinones have an amino group so cation-exchange sorbents are suitable for this type of compounds, which have been previously demonstrated by other authors who have used this kind of sorbents to extract these compounds from environmental and biological samples [9, 11]. Their pKa values are between 7.3 and 8.2, due to the aforementioned amino group, which means that working at values of pH lower that these pKa values, cationic interactions might be present with the sorbent. In this study, two different cation-exchange sorbents were compared, a weak one (Oasis WCX) and a strong one (Oasis MCX). These experiments were performed first with standards and then with urine by using LC-DAD. Once the optimal protocols were achieved, they were evaluated by LC-HRMS.

Oasis WCX

The initial protocol for Oasis WCX was conditioning with 5 mL of MeOH followed by 5 mL of phosphate buffer solution at pH 7 (0.10 M of NaH_2PO_4 and 0.06 M of Na_2HPO_4), loading with 5 mL of the buffer solution (pH 7) containing the cathinones at a concentration of 2 mg L^{-1}, washing with MeOH and eluting with 5% HCOOH in MeOH. At pH 7 all the cathinones are in their cationic form and this allows the interaction with the sorbent since the carboxyl group is also deprotonated. Fixing the conditioning and the loading steps, the clean-up and the elution steps were optimised to achieve the highest recoveries with the minimal elution solvent volume. For this, different volumes of elution solvent were tested without the clean-up step. In particular, values of 2 mL, 4 mL and 5 mL of 5% HCOOH in MeOH were evaluated. It was observed that 2 mL of 5% HCOOH in MeOH were enough to ensure the complete elution of all the cathinones under study with recovery values between 90% and 104%.

After optimising the elution step, the clean-up was evaluated with a mixture of urine:phosphate buffer solution (pH 7) (1:1, *v/v*). The aim of this step is to break the non-specific interactions between the interferences and the sorbent without breaking the ionic interactions (specific) between the analytes of interest and the sorbent. Therefore, the composition and volume of the washing solvent were optimised. MeOH was tested first due to the good results obtained in the determination of cathinones in environmental samples [11]. In particular, different volumes were evaluated: 2 mL, 4 mL and 5 mL of MeOH. Unfortunately, even with the lowest volume of MeOH used, all the analytes were eluted. One possible explanation is that urea, which is present at high concentrations in urine, might compete with the cathinones at the active ionic sites and might displace the interactions with them. Therefore, a change in the washing solvent was made and clean-ups with MeOH:H_2O (75:25, *v/v*) and H_2O were tested with the same volumes previously tested for MeOH. In the case of MeOH:H_2O (75:25, *v/v*), the analytes were partially lost and in the case of H_2O, there were no losses. Therefore, 2 mL of H_2O were enough to clean most of the interferences such as salts and ionic species. From the obtained results, it can be concluded that if all the analytes are eluted with 2 mL of MeOH, then the acid (5% HCOOH) is not necessary in the elution solvent since the analytes are probably only retained by reversed-phase interactions.

After this, a mixture of urine:phosphate buffer solution (pH 7) (1:1, *v/v*) spiked with cathinones at a concentration of 2 mg L^{-1} was loaded into the cartridge, then it was washed with 2 mL of water and finally, the analytes were eluted with 2 mL of MeOH. The extraction yields using the optimised protocol for Oasis WCX were between 84% and 105%.

Oasis MCX

The initial protocol for the Oasis MCX cartridge was to use 5 mL of MeOH and 5 mL of phosphate buffer solution at pH 6 (0.15 M of NaH_2PO_4 and 0.05 M of Na_2HPO_4) for conditioning, 5 mL of the buffer solution (pH 6) containing cathinones at a concentration of 2 mg L^{-1} as loading step, MeOH for the clean-up and 5% of NH_4OH in MeOH as the elution solvent. This pH, which makes it possible for all cathinones to be in their cationic form, has been previously studied in urine and values of pH 3 and 6 have been compared. Better results were obtained at pH 6 when urine samples were analysed. As before, with the initial conditioning and loading steps fixed, the washing and elution were optimised by using LC-DAD to achieve the highest recoveries using the minimum volume of solvent.

In this case, 2 mL, 4 mL and 5 mL of 5% of NH_4OH in MeOH were examined in the elution step without the clean-up step. It was observed that 2 mL was enough to elute the analytes with recoveries between 90% and 105%. Higher elution volumes (4 mL and 5 mL) did not improve the recoveries.

The washing step was evaluated with urine and volumes of 2 mL, 4 mL and 5 mL of MeOH were tested for this step. It was observed that with 2 mL of MeOH there were no losses. However, with 4 mL it could be observed that some analytes were lost and 2 mL of MeOH was chosen as the optimal washing solvent. It has been previously observed that when a 60 mg Oasis MCX cartridge in the analysis of doping compounds, 2 mL of MeOH are suitable for the washing step in urine samples [37].

Once the protocol had been optimised, a mixture of urine:phosphate buffer solution (pH 6) (1:1, *v/v*) spiked with cathinones at a concentration of 2 mg L^{-1} was loaded. The extraction recoveries using the optimised protocol for Oasis MCX were between 84% and 101%.

Comparison between Oasis WCX and Oasis MCX

As it can be seen in Fig. 1, both sorbents achieved recoveries higher than 80% when they were tested by LC-DAD. Due to the acceptable results obtained for the two sorbents in terms of extraction, they were compared by evaluating the clean-up step, the recoveries of the extraction procedure and the matrix effect (% ME) by using LC-HRMS. As it is well known that when an MS detector is used, the matrix effect must be considered because it can cause an enhancement or a suppression of the signal.

Firstly, the performance of the two SPE sorbents was compared by loading 5 mL of the mixture urine:buffer solution (pH 6 with Oasis MCX or pH 7 with Oasis WCX) (1:1, *v/v*) containing cathinones at a concentration of 40 ng mL^{-1} without the clean-up step and then comparing these results with the ones obtained with a clean-up step. A higher % ME was obtained for Oasis WCX (between -29% and -73%), while for Oasis MCX it was between -27% and 50%. Both sorbents had high values of % ME, which means that a clean-up in the SPE is necessary to reduce this effect. Table 2 shows the values of % ME with and without the clean-up step. A reduction of the % ME can be observed in both cases if a clean-up step is applied. Better results of % ME were obtained for Oasis MCX with the clean-up step, and therefore it was selected as the optimal sorbent for its better clean-up with MeOH.

In this experiment, the SPE extracts were evaporated to dryness under a gentle stream of N_2. Cathinones are

volatile in their base form and it is necessary to neutralise the basic extract solution by adding 100 µL of 1% HCl in MeOH before the evaporation [38]. Finally, after the solution had been reconstituted with 1 mL of mobile phase, it was filtered through a 0.45 µm PTFE filter and injected into the LC-HRMS.

Method validation

The analytical performance of the method developed was investigated under optimal conditions by evaluating the % R_{app}, % ME, linear range, the MDLs, MQLs, repeatability (intra-day) and reproducibility (inter-day). The precision parameters were studied at two concentration levels, 1 ng mL^{-1} (low level) and 40 ng mL^{-1} (high level). Table 3 shows the values at the two levels of concentration. Moreover, a non-spiked urine sample was included in the analysis to subtract any analyte signal present in the samples.

Values of % R_{app} at high concentration were between 69% and 82%, and these values were mainly affected by the ME. Values at low concentration were between 75% and 125% and as stated before, they were mainly affected by the ME. In general, for both concentration levels a suppression of the signal was observed (between -8% and -28%). However, 4-MEC and alpha-PVP showed enhancement at the low concentration (12% and 21% respectively). These values of % ME are considered acceptable.

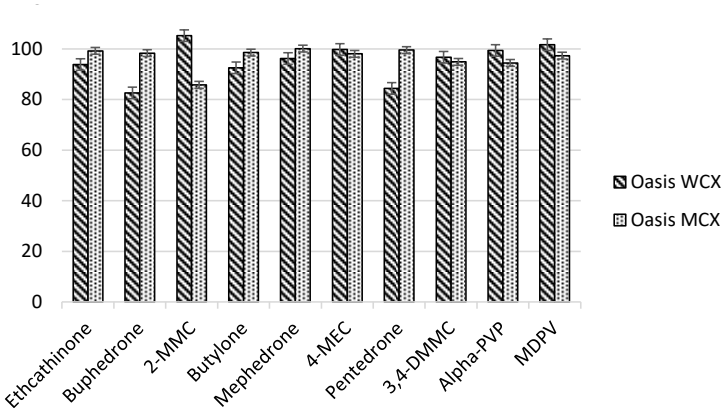

Figure 1. Comparison of the recoveries obtained by SPE when 5 mL of a standard sample spiked at 40 ng mL^{-1} of each analyte were percolated through Oasis WCX and Oasis MCX sorbents and analysed by LC-DAD.

Table 2. % Matrix effect of Oasis WCX and Oasis MCX with and without clean-up step in the SPE from a mixture of cathinones at 40 ng mL^{-1} using LC-HRMS and the % RSD values in brackets.

n=3	% ME Oasis WCX		% ME Oasis MCX	
40 ng mL^{-1}	Without clean-up	With clean-up	Without clean-up	With clean-up
Flephedrone	-73 (6)	-50 (5)	-50 (4)	-21 (2)
Ethcathinone	-46 (8)	-34 (4)	-48 (3)	-20 (1)
Buphedrone	-35 (8)	-28 (7)	-39 (5)	-12 (2)
2-MMC	-44 (10)	-36 (7)	-27 (5)	-9 (3)
Butylone	-29 (11)	-24 (8)	1 (10)	-12 (2)
Mephedrone	-36 (4)	-28 (9)	-37 (7)	-22 (1)
4-MEC	-50 (6)	-36 (8)	-49 (9)	-10 (12)
Pentedrone	-41 (9)	-34 (7)	-40 (6)	-20 (2)
3,4-DMMC	-42 (7)	-31 (5)	-40 (4)	-15 (2)
Alpha-PVP	-49 (7)	-34 (7)	-49 (8)	-10 (1)
MDPV	-49 (8)	-34 (5)	-45 (5)	-22 (4)

Although these ME values are similar to the ones previously obtained by Concheiro et al. [9], who used the SOLA SCX sorbent, better recoveries of the extraction procedure can be observed in our case.

As the ME using Oasis MCX was higher than -20% in some cases at both concentration levels (1 ng mL^{-1} and 40 ng mL^{-1}), it was decided to use a matrix-matched calibration curve to deal with the ME. The linear range was from MQL to 100 ng mL^{-1} for most compounds or to 150 ng mL^{-1} for 4-MEC, pentedrone, 3,4-DMMC, alpha-PVP and MDPV, in all cases obtaining r^2 values higher than 0.999.

The MDLs were between 0.04 ng mL^{-1} and 0.16 ng mL^{-1} while the MQLs were 0.2 ng mL^{-1} for all the compounds. Thus, the developed method achieved lower MDL and MQL values than a previously published study in which a similar strategy was reported; however, in that case, the main difference was the use of another kind of cation-exchange sorbent (Sola SCX) [9]. They achieved recoveries between 79% and 117% and detection and quantification limits of 0.25 ng mL^{-1} and 0.5 ng mL^{-1}, respectively when a Q-Orbitrap instrument was used. If the method is also compared with other HRMS methods, which used salting-out liquid-liquid extraction (SALLE) [12] or liquid-liquid extraction (LLE) [30], it can be observed that lower MDL and MQL values are obtained with the present method.

Repeatability and reproducibility were studied at both levels of concentration by analysing five spiked samples on the same day and on five different days. Values at 1 ng mL^{-1} in terms of relative standard deviation (% RSD) were between 1% and 13% for repeatability and 2% and 10% for reproducibility.

To prove the accuracy of the method, as we could not apply the method to urine samples from drug users, it was used to analyse different blind samples and the concentration of the cathinones were obtained by the matrix-matched calibration curve. All the blind samples showed similar results in terms of bias, which were lower than 10% in all cases. For example, one sample was spiked by a colleague at 46 ng mL^{-1} and after the procedure was completed, the concentrations obtained were between 45 and 53 ng mL^{-1} for the different cathinones, and the average accuracy in terms of bias was 7%, which shows acceptable results and proves the reliability of this method. An example of a chromatogram of a blind sample can be observed in Fig. 2.

Conclusions

A method based on SPE using a strong cation-exchange sorbent (Oasis MCX) followed by LC-HRMS using Orbitrap as analyser was successfully developed and validated for determining synthetic cathinones in urine.

Table 3. Validation parameters of the method based on SPE with Oasis MCX followed by LC-HRMS for the determination of synthetic cathinones in urine.

Analyte	% ME[a] (n=3)	% R_{app}[a] (n=5)	% ME[b] (n=3)	% R_{app}[b] (n=5)	MDLs[c]	Linear range[c]	Repeatability[a] % RSD (n=5)	Reproducibility[a] % RSD (n=5)
Flephedrone	-24	73	-21	73	0.08	0.2-100	2	3
Ethcathinone	-21	84	-20	76	0.10	0.2-100	5	4
Buphedrone	-14	90	-12	85	0.10	0.2-100	7	6
2-MMC	-10	93	-9	88	0.10	0.2-100	7	6
Butylone	-8	96	-12	84	0.12	0.2-100	9	7
Mephedrone	-26	81	-22	76	0.08	0.2-100	13	10
4-MEC	12	115	-10	87	0.04	0.2-150	4	4
Pentedrone	-22	76	-20	74	0.04	0.2-150	6	5
3,4-DMMC	-18	76	-15	79	0.04	0.2-150	11	9
Alpha-PVP	21	125	-10	87	0.16	0.2-150	1	2
MDPV	-28	82	-22	69	0.06	0.2-150	7	6

[a] spiked at 1 ng mL^{-1} ; [b] spiked at 40 ng mL^{-1} ; [c] concentration in ng mL^{-1}

Two different cation-exchange sorbents were studied to extract the cathinones from urine, a weak one (Oasis WCX) and a strong one (Oasis MCX). Oasis MCX achieved excellent recoveries (around 100%) and acceptable levels of MEs (reduced if a clean-up step was applied). The present method achieved very low MDLs and MQLs, which are suitable for detecting this kind of compounds in urine at the usual concentration levels found. Thus, the method developed is a promising tool for forensic and toxicological analyses.

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Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

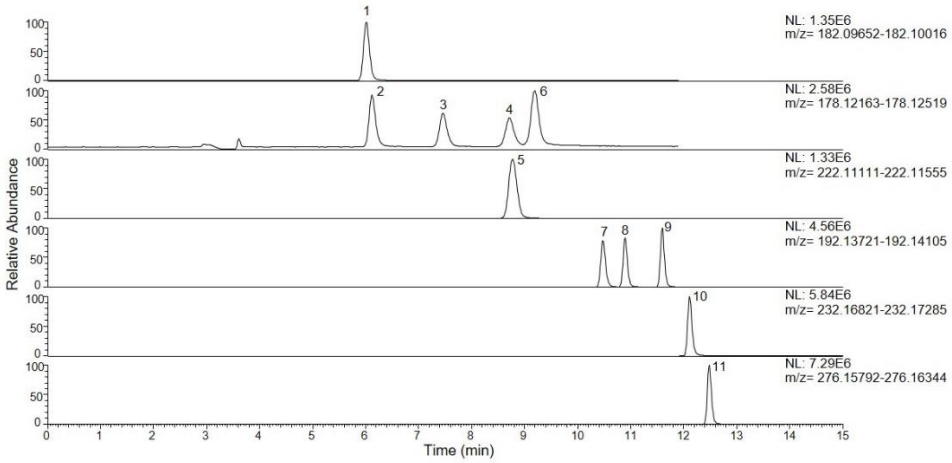

Figure 2. Chromatogram of a urine blind sample analysed by SPE/LC-MS/MS under the optimal conditions. 1: flephedrone, 2: ethcathinone, 3: buphedrone, 4: 2-MMC, 5: butylone, 6: mephedrone, 7: 4-MEC, 8: pentedrone, 9: 3,4-DMMC, 10: alpha-PVP and 11: MDPV.

Informed consent was obtained from all individual participants included in the study.

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3.1.2. A fast analytical method for determining synthetic cathinones in oral fluid by liquid chromatography-tandem mass spectrometry

UNIVERSITAT ROVIRA I VIRGILI
DETERMINATION OF DRUGS OF ABUSE IN URINE AND ORAL FLUID SAMPLES
Sergi Pascual Caro

A fast analytical method for determining synthetic cathinones in oral fluid by liquid chromatography-tandem mass spectrometry

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Abstract

In this paper we present a method for simultaneously determining eleven synthetic cathinones in oral fluid by liquid chromatography-tandem mass spectrometry. Synthetic cathinones, a wide variety of which are available on the market, are constantly evolving. It is therefore important to provide efficient methods for determining cathinones in different matrices. A common matrix for detecting recent drug intake is oral fluid, which can easily be collected using one of numerous commercial devices. Most methods aimed at determining drugs in biological samples such as oral fluid require labour intensive and time-consuming sample-preparation steps. However, the pre-treatment of complex samples is often a challenge in the development of a method. For this reason, in this paper we present a simple, easy-to-handle alternative that uses a Salivette® device and pre-treats the sample in the same device. Matrix-matched calibration curves were used to cover the concentration range at which these substances are usually present in oral fluid from drug consumers. The method detection limits ranged from 0.003 and 0.03 ng/g and the method quantification limits were set at 0.075 ng/g. This is a simple, rapid and sensitive method with good potential for determining recent drug consumption in oral fluid.

Keywords: *Synthetic cathinones · Liquid chromatography-tandem mass spectrometry · Biological samples · Oral fluid· Salivette®*

Introduction

The last few years have seen the emergence of new psychoactive substances (NPS) as well as a greater awareness of those substances due to their associated health risks (1, 2). The United Nations Office on Drugs and Crime (UNODC) began monitoring NPS in 2009. Between 2009 and 2015 a sharp increase in their use was reported. Since then they have remained one of the most commonly used drugs (2). NPS include, for example, synthetic cannabinoids, synthetic cathinones, and phenethylamines. Synthetic cathinones are the second largest group of NPS, accounting for 30% of NPS seized globally (2). According to a 2019 report, 138 synthetic cathinones are currently monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (3).

Synthetic cathinones are commonly known as “bath salts” and come from the molecule cathinone, which is found in the Catha edulis plant. They were first produced as antidepressants and anorectic agents for clinical use. However, they began to be used as recreational drugs and are now a legal alternative to illicit drugs such as ecstasy and cocaine (4). Their structure is similar to that of amphetamines (the main difference between them is the ketone group at the β position of the amino chain in the cathinones). For this reason, they are often sold as cheap alternatives to amphetamines. Synthetic cathinones have been shown to cause addiction and to produce a range of effects, including paranoia and anxiety (5). However, they are difficult to monitor. This is because new derivates are usually synthetized by changing the structures of existing synthetic cathinones and by the time one substance is controlled another is launched onto the market.

In recent years, drug analysis has become a great concern for society. Biological matrices such as urine, blood, hair, oral fluid (OF) and sweat have therefore been studied to detect or monitor drug use (6–8). Of these matrices, OF is considered a good alternative to urine and blood thanks partly to its easy, non-invasive and observable sample collection, which makes adulteration difficult. In OF, the concentration of the parent drug (from low ng/mL to high ng/mL (9)) is usually higher than in other matrices, which enables the detection of recent drug use. Also, since numerous sampling devices are available, on-site sampling is possible (6, 10, 11).

The methods developed in the literature for determining drugs in OF usually involve three main steps, i.e. sampling, sample pre-treatment, and chromatographic analysis.

With regard to sampling, saliva can be obtained by several approaches. These include transferring the sample from the oral cavity to a recipient

(directly, by spitting); aspirating the saliva using a vacuum tube with a passive drool (i.e. allowing the saliva to pool at the bottom of the mouth and collecting it with a straw); or placing a swab in the mouth to retain the saliva (12–14). The latter is the most common sample-collection strategy, and numerous devices have been developed based on leaving a swab or absorbent pad in the mouth for a short time (6). Most of these devices contain a solid base, which is usually a small piece of cotton or polyester for absorbing the saliva and a conical tube for centrifuging and recovering the collected saliva. The literature on determining synthetic cathinones in OF is rather limited. However, examples exist of studies using sampling devices such as Quantisal™ (15–17), Salivette® (18), Intercept® (19), Oral-Eze™ (17) and DCD5000® (20). The main advantage of these devices is that after sample collection, the swab is centrifuged and the OF collected directly in the same device. Each of these commercial devices has its own recommended operation conditions and design. The collection device is therefore a significant variable in OF analysis because such differences can greatly affect the efficiency of the collected OF (21).

After the sample is collected, the sample pre-treatment methods reported in the literature usually use solid phase extraction (SPE) (15–17, 22), liquid-liquid extraction (LLE) (19), microextraction by packed sorbents (MEPS) (23), or ultrasound-assisted dispersive liquid-liquid microextraction (US-DLMME) (18) to extract the analytes from the OF sample, clean the matrix, and remove any interference.

Finally, gas chromatography coupled to mass-spectrometry (MS) was usually used in the past as a confirmation technique for these compounds. However, this technique has several drawbacks compared to liquid chromatography (LC), including the lack of the detection of polar and thermally labile analytes and the need to introduce a derivatization process before analysis (15, 18, 24). Synthetic cathinones in OF are therefore normally determined by LC in combination with MS or tandem MS (MS/MS).

In this study we used a Salivette® device to collect the OF for determining synthetic cathinones. The studies that we reported earlier that employed the same type of sampling device also used an extraction step after collecting the sample (15–19, 22, 23). However, the procedures for doing so are laborious because they require various organic solvents and long pre-treatment times. For example, Férnandez et al. (18) used a Salivette® device to collect the sample and then used US-DLLME. For that procedure, the OF was diluted in water, MeOH was added, and the mixture was then centrifuged for five minutes. The

supernatant was then mixed with water at pH 8 and chloroform was added. The solution was then placed in an ultrasonic bath for five minutes and centrifuged for five more minutes. Finally, the extraction solvent was evaporated to dryness, reconstituted and injected into the UPLC-MS/MS system.

Given the tedious procedures involved in the above strategies, our main aim in this paper was to simplify sample pre-treatment by avoiding several steps and achieve a fast analytical method for determining a group of synthetic cathinones in OF samples by LC-MS/MS.

Experimental

Standards and materials

Cathinone standards were purchased from LGC Standards (Luckenwalde, Germany). The synthetic cathinones selected were: 4-fluoromethcathinone (flephedrone), N-ethylcathinone (ethcathinone), buphedrone, 2-methylmethcathinone (2-MMC), butylone, 4-methyl-methcathinone (mephedrone), 4-methylethcathinone (4-MEC), beta-ethylmethcathinone (pentedrone), 3,4- dimethylmethyl-cathinone (3,4-DMMC), alpha-pyrrolidinovalerophenone (alpha-PVP) and methylenedioxypyrovalerone (MDPV). Two deuterated cathinones (mephedrone-D_3 and alpha-PVP-D_8) were used.

Individual stock standard solutions at 100 mg/L, 1000 mg/L and 2000 mg/L were prepared in methanol (MeOH). From these, the mixture solution of all synthetic cathinones in MeOH at 1 mg/L was prepared and further diluted to appropriate concentrations with the mobile phase to prepare the working solutions. All of these solutions were stored in the freezer at -20ºC.

Acetonitrile (ACN) for LC-MS and water for LC-MS were obtained from Scharlab (Barcelona, Spain). MeOH was purchased from J.T. Baker (Deventer, The Netherlands). Formic acid (HCOOH) and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Ultrapure water was obtained using a water purification system (Merck Millipore, Darmstadt, Germany).

Salivette® devices were obtained from Starstedt (Nümbrecht, Germany). A Hettich Universal 32R centrifuge machine from Hettich (Tuttlingen, Germany) was used to centrifuge the collector devices.

HPLC-MS/MS

An Agilent model 1200 series LC coupled with an Agilent 6460 series triple quadrupole mass spectrometer with an electrospray ionisation (ESI) interface from Agilent Technologies (Waldbronn, Germany) was used for the

analysis. Agilent MassHunter Workstation Software version B.09.00 was used for instrumental control and data analysis.

Chromatographic separation was achieved using a Luna Omega 5 µm Polar C_{18} (150 mm x 4.6 mm, 5 µm) from Phenomenex (Torrance, CA, United States) with a Security Guard from Phenomenex. The mobile phase flow rate was set at 0.6 mL/min and the sample volume injected was 20 µL. The mobile phase was A: 0.1% HCOOH in H_2O and B: 0.1% HCOOH in ACN in gradient mode. The gradient began at 15% B. This was maintained for 5 minutes, then increased to 35% in 5 minutes, then to 80% in 4 minutes and to 100% in 1 minute. This was maintained for 2 minutes before the gradient was returned to the initial conditions in 1 minute and maintained for 4 minutes.

The LC-MS/MS parameters were optimised by injecting each compound at 1 mg/L in a mixture of H_2O:MeOH (50:50, *v/v*). The acquisition was performed in Multiple Reaction Monitoring (MRM) mode in positive polarity. The cathinones were divided into five windows depending on their retention times (Rt): flephedrone and ethcathinones; buphedrone; 2-MMC, butylone and mephedrone; 4-MEC and pentedrone; and 3,4-DMMC, alpha-PVP and MDPV. The optimised source parameters were as follows: gas temperature, 350ºC; gas flow rate, 13 L/min; nebulizer pressure, 60 psi; and capillary voltage 2500V. The fragmentor was between 100 and 125 V, and the collision energy (CE) was between 8 and 25 eV. For the MRM mode, the two most intensive transitions between the parent ion and the product ions were selected (Table 1).

Oral fluid collection and preparation

OF samples were collected using a Salivette® device in accordance with the manufacturer's recommendations (25). The cotton swab of the Salivette® was placed in the mouth, chewed and rolled around for 1 minute. The swab was then returned to the tube and centrifuged for 1 minute at 8000 rpm. The saliva dropped to the bottom of the tube and was discarded. Then, 1 mL of MeOH was added to the swab, which was again centrifuged for 1 minute at 8000 rpm. This methanolic solution was collected and the procedure was repeated. The two methanolic solutions were combined and 100 µL of 1% HCl in MeOH (1:1*, v/v*) were added to the final solution. This was evaporated to dryness under a gentle stream of nitrogen, reconstituted with 500 µL of mobile phase at initial chromatographic conditions, filtered through a 0.45 µm PTFE filter, and finally injected into the LC-MS/MS system.

Table 1. MRM parameters of the synthetic cathinones under study

Compound	Rt (min)	Precursor ion	Product ion 1	CE 1 (eV)	Product ion 2	CE 2 (eV)	Product ion 3	CE 3 (eV)	Fragmentor voltage (V)
Flephedrone	6.90	182.1	164	15	149	15	123	15	100
Ethcathinone	6.96	178.1	160	12	132	14	105	18	100
Buphedrone	8.76	178.1	160	10	131	15	132	15	100
2-MMC	10.51	178.1	160	8	145	15	119	15	100
Butylone	10.78	222.1	174	14	204	10	191	10	100
Mephedrone	10.92	178.1	160	8	145	15	119	15	100
4-MEC	12.10	192.1	174	10	146	16	145	18	100
Pentedrone	12.48	192.1	174	10	132	16	91	18	100
3,4-DMMC	13.26	192.1	174	10	159	15	133	15	125
Alpha-PVP	13.73	232.2	91	22	105	25	126	25	125
MDPV	14.21	276.2	126	25	135	25	175	20	125

Method validation

The method was validated following the European guidelines for workplace drug testing in oral fluid (26). It was studied in terms of linearity, sensitivity, instrumental detection and quantification limits (IDLs and IQLs), method detection and quantification limits (MDLs and MQLs or LODs and LOQs), precision in terms of repeatability (intra-day) and reproducibility (inter-day), matrix effect (ME), apparent recoveries (R_{app}), selectivity, stability and accuracy. For analyte confirmation, the retention time had to correspond to a tolerance of ±2.5% and the ion ratio had to correspond to a tolerance of ±20% (27). Different units were used in the method: ng/mL was used when working with standard samples, ng/g was used to express the analyte concentration in OF, since in this case saliva was weighed and this parameter changed from sample to sample. Finally, ng was used to express the amount of analyte in OF for the calibration curves since this depends on the OF weighed. The Salivette® device was weighed before and after sampling.

The instrumental linearity was studied in the same day per triplicate with a mix of cathinone neat standards at different calibrator concentrations: 0.1, 0.2, 0.5, 1, 2, 10, 25, 40, 50, 75, 100, 200 and 300 ng/mL by evaluating the coefficient of determination (r^2) and the sensitivity of the curve. IDLs were

defined as the lowest detectable concentrations with a signal-to-noise ratio (S/N) higher than 3. IQLs were the lowest point in the calibration curve with a S/N higher than 10.

In the case of the method linearity, it was studied at 0.075, 0.1, 0.2, 0.5, 1, 4, 10, 25, 40, 75, 100 and 125 ng of cathinone in OF and in the same way as the instrumental linearity, by evaluating the r^2 and the sensitivity of the curve. For MDLs and MQLs, the same criteria as for the instrumental limits were considered, but in this case for extracted specimens. The R_{app}, ME, repeatability and reproducibility values were studied using five replicates (n=5) at low and high levels of concentration spiked in OF, i.e. 1 ng/g and 40 ng/g (expressed as nanograms of cathinone per grams of weighted OF). These latter two terms were calculated in terms of relative standard deviation (%RSD). The repeatability was studied by analysing five replicates the same day and the reproducibility by analysing one replicate during five consecutive days.

The %ME was calculated using the following formula:

$$\%ME = \left[\left(\frac{C_{spiked}}{C_{STD}}\right) x100\right] - 100$$

(Equation 1)

where CSTD is the concentration of an analyte standard sample injected directly into the LC-MS/MS system and C_{spiked} is the analyte concentration obtained from a blank of an OF sample spiked with the analyte at the same concentration as C_{STD} before injecting it into the LC-MS/MS system. Negative %ME values indicate signal suppression, while positive values indicate signal enhancement.

The $\%R_{app}$, defined as the recovery of the whole method (from chewing of the cotton swab to LC-MS/MS injection), was calculated as follows:

$$\%R_{app} = \left(\frac{C_{spiked}}{C_{STD}}\right) x100$$

(Equation 2)

The selectivity and specificity were evaluated by studying the possible endogenous and exogenous interferences from 15 different saliva sources. The autosampler stability was studied at low and high level of concentration by remaining the samples in the autosampler at 10°C for 30 h and reanalysing them (n=5).

To study the method's accuracy in terms of error, three blind samples were analysed using the same method as with the other samples. To obtain these samples, the swab was chewed and then it was spiked with a mix of cathinones by a member of the laboratory staff before all the experimental procedure. The ng of the cathinones was then calculated with the matrix-matched calibration curves and divided by the amount of weighed OF. This enabled the cathinone concentration in the sample to be

determined. The values obtained were compared to the real ones and accuracy was calculated from Equation 3.

$$\%\text{Accuracy}=\left|\left(\frac{C_{spiked}-C_{calculated}}{C_{spiked}}\right)x100\right|$$

(Equation 3)

Results and discussion

Instrumental optimisation

The chromatographic separation conditions used in this study were the same as those used in a previous study by our research group when determining the same group of synthetic cathinones in urine (28).

For the MS/MS system, the various conditions – including MRM transitions, collision energies (CE) and fragmentor voltages – were individually optimised for all analytes by using individual standard solutions of the cathinones under study and a mixture of them at a concentration of 1 ng/mL in H_2O:MeOH 50:50 (*v/v*). The individual standard samples were injected in order to choose the precursor ions for each cathinone in positive mode since these are basic compounds. The fragmentor voltage was then optimised between 50 and 200 V. With the fragmentor voltage fixed, the collision energies were studied for all compounds between 0 and 30 kV and the product ions were found for the optimal collision energies. The two most abundant transitions of each cathinone were selected to enable their correct identification. Once the MRM method was created, the source conditions (capillary voltage, gas flow rate, gas temperature and nebuliser) were optimised using the Source optimiser software. The fragmentor voltage was then re-evaluated between the voltages previously obtained (50-125 V). With the final optimal values of the fragmentor voltage, the following five segments were created according to retention times and fragmentor values of the cathinones: flephedrone and ethcathinone; buphedrone; 2-MMC, butylone and mephedrone; 4-MEC and pentedrone; 3,4-DMMC, alpha-PVP and MDPV.

Sample collection and pre-treatment optimisation

As we mentioned in the Introduction, a previous study determined a group of 20 drugs of abuse, including cathinones, using a Salivette® device as the OF collector [18]. That study included an extraction step based on US-DLLME before chromatographic analysis of the samples. Although the results were promising in terms of the low sensitivity achieved, sample pre-treatment considerably increased overall analysis time. To simplify the sample pre-treatment step, we performed this pre-treatment in the same collector device without any additional extraction step.

To do so, we focused on the parameters that may affect Salivette® performance (mainly those referring to extraction of the analyte from the oral fluid). The literature provides several examples with this kind of sampling device used for various purposes and there is no homogeneous way to proceed (18, 29). For example, to analyse cannabinoids, Mazina et al. (29) used the Salivette® to collect the sample and, after the swab was chewed and centrifuged for 1 minute, they discarded the centrifugate. They then added 1 mL of ACN to the swab, centrifuged it again and analysed the final solution using capillary electrophoresis (CE). However, other strategies, such as that by Fernández et al. (18), used the same device to collect the sample and kept the first centrifugate for the US-DLLME procedure. For these reasons, in this study we optimised the parameters that may affect sampling and analyte extraction, i.e. solvent type and volume, centrifugation time and speed, and the number of times the solvent is added to the swab and centrifuged. Also, due to discrepancies in the literature, we evaluated whether to keep or discard the first centrifugate of saliva.

Our starting pre-treatment conditions were based on the general instructions from Salivette® (25). Once the swab was chewed, it was centrifuged and the particles and mucus collected in the special tip of the Salivette® tube. A solvent was then added to desorb the analytes from the swab. In all experiments, the saliva samples were spiked at a concentration of 150 ng/g with a mixture of synthetic cathinones and five replicates of OF samples from the same person were analysed (n=5).

The first parameter studied in relation to sample pre-treatment was the solvent used to desorb the analytes from the swab. MeOH, ACN and H_2O were compared. Recoveries were slightly better (2–15% higher) for MeOH than for ACN and much better (7–44% higher) than for H_2O as it can be observed in Figure 1. We also studied solvent volume (at 0.5 mL and 1 mL). The final volume was set at 1 mL since this was the optimal volume for soaking the whole swab. We therefore selected 1 mL of MeOH as the optimal solvent type and volume. Then we tested three centrifugation times: 1, 2 and 3 minutes. Since 2 and 3 minutes did not improve recoveries, we selected 1 minute as the centrifugation time. Also, since centrifugation speeds of 8000 rpm and 9000 rpm achieved similar recoveries, we selected 8000 rpm. We also evaluated the number of times (one, two or three) that 1 mL of MeOH was added to the swab and centrifuged. Here we found that recoveries with two repetitions were much higher than those with a single repetition (15–52% higher). However, a third repetition led to a minimal increase (roughly 5% higher) and was therefore discarded.

Our final procedure was therefore established as two centrifugations following the addition of 1 mL of MeOH for each.

As mentioned earlier, we also studied the effects of keeping or discarding the first centrifugate of saliva (the one without solvent) on recoveries and ME by analysing five replicates of saliva samples from one person (n=5). Considering the good $\%R_{app}$ achieved discarding the first centrifugation (Figure 2) and that %ME were higher when the first centrifugate was kept (up to-32%) than when it was discarded (up to-19%) (Figure 3), the first centrifugate of saliva was discarded. Finally, to preconcentrate the analytes, we considered an evaporation step with the two methanolic solutions obtained for the saliva. We evaluated the losses in this and in the filtration step after the entire extraction process had been conducted. This was because in a previous study conducted by our group, in which the same synthetic cathinones were evaporated in a basic methanolic solution (5% NH_4OH in MeOH), 100 µL of 1% of HCl in MeOH were added before the evaporation step in order to prevent losses (28). In the present study the composition of the final solution is different from that of the previous study (since a methanolic solution, rather than a basic one, is obtained), so we studied evaporation with and without adding 1% of HCl in MeOH. Our results showed that losses were significant when the acidic solution was not added (up to 80%) but considerably lower (up to 9%) with the addition of 100 µL of HCl 1M. The acidic methanolic solution was therefore added before the evaporation step.

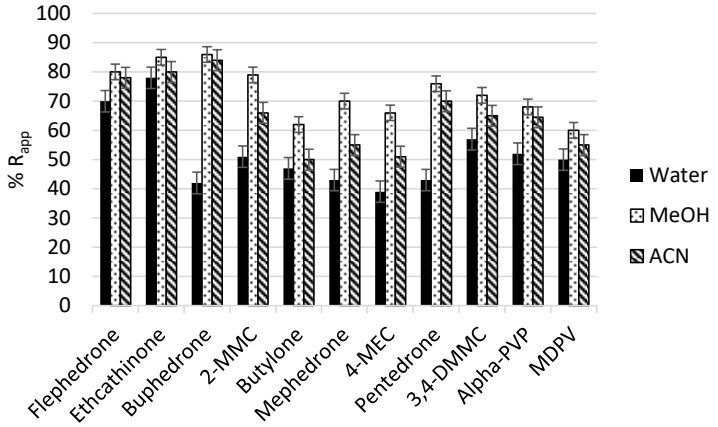

Figure 1. Comparison of $\%R_{app}$ obtained when different solvents were tested in the sample pretreatment of a sample containing a mixture of cathinones at 150 ng/g and analyzing in five replicates.

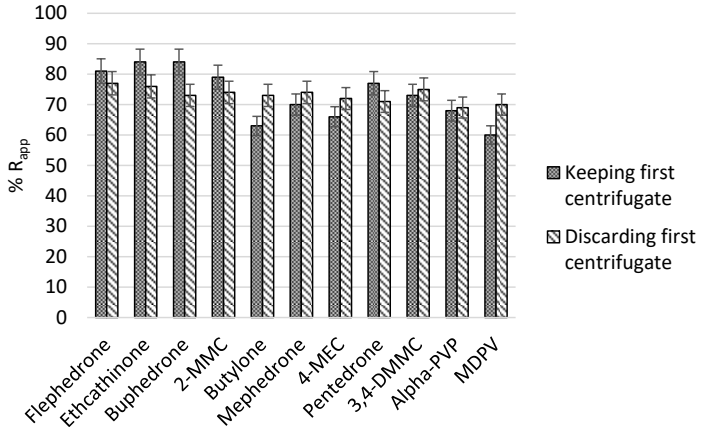

Figure 2. Comparison of $\%R_{app}$ between keeping or discarding the first centrifugate of saliva in the sample pretreatment. These values corresponded to the analysis of five replicates of saliva containing the cathinones at 150 ng/g.

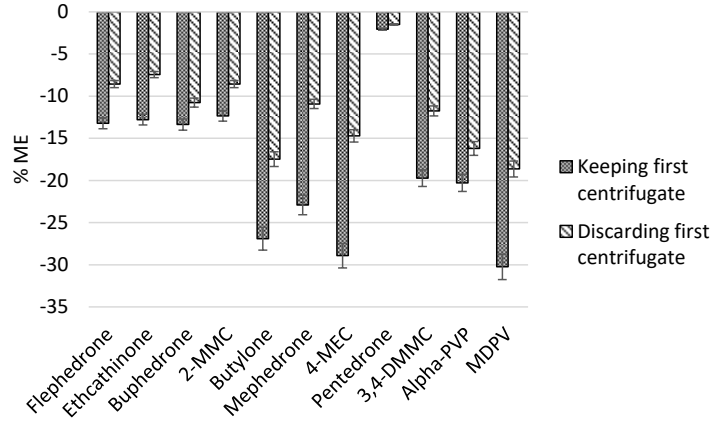

Figure 3. Comparison of %ME between keeping or discarding the first centrifugate of saliva in the sample pretreatment. These values corresponded to the analysis of five replicates of saliva containing the cathinones at 150 ng/g.

The solution was then diluted with 500 µL of mobile phase under the initial chromatographic conditions and filtered through a 0.45 µm PTFE filter

Method validation

Instrumental linearity ranged from the IQL to 150 ng/mL for most cathinones and from the IQL to 250 ng/mL for buphedrone, mephedrone and pentedrone. The IDLs ranged from 0.001 to 0.010 ng/mL and the IQLs ranged from 0.01 to 0.05 ng/mL.

To validate the method, we studied the addition of two deuterated standards (mephedrone-D_3 and alpha-PVP-D_8) as surrogates. Due to their structural similarity, mephedrone-D_3 was applied to most cathinones and alpha-PVP-D_8 was applied to alpha-PVP and MDPV after the swab was chewed and the first centrifugation performed. However, different behaviours were observed even for one cathinone and its deuterated (around 20% recovery deviation). For this reason, the surrogate standards were not included in the procedure, and matrix-matched calibration curves were performed to compensate for any losses in the process.

The parameters for method validation were studied at low and high levels of concentration in the OF (1 ng/g and 40 ng/g, respectively). Analysis of 15 blank OF samples from different laboratory staff members showed no endogenous or exogenous interferences at the same retention times of the synthetic cathinones, demonstrating good selectivity of the developed method. In particular, as exogenous interferences we evaluated cocaine and a group of amphetamines. Table 2 shows the method parameters at low and high levels of concentration. The best method calibration model was adjusted to two linear calibration curves with r^2 higher than 0.999, i.e. from 0.075 to 4 ng and from 4 to 75 ng. The MDLs in OF ranged from 0.003 to 0.03 ng/g, while the MQLs were 0.075 ng/g for all compounds. Values of $\%R_{app}$ at low (1 ng/g) and high (40 ng/g) concentration levels in OF ranged from 50 to 60% and from 61 to 66%, respectively. For %ME, the values at low and high levels ranged from -14 to -21% and from -5 to -10%, respectively. Although no cleaning step was performed, the values of ME showed that there was no great interference of the matrix and that no further step was required. Method repeatability and reproducibility were also studied at both concentration levels, and RSDs were below 20% which are considered acceptable. The stability study showed values of deviation between 1 and 8% for the samples analysed, demonstrating that the analytes were stable at 10°C during 30 h and did not present any evidence of degradation during sample processing.

Table 2. Validation parameters for the determination of synthetic cathinones in OF by LC-MS/MS

Analyte	MDLs[a] (LODs)	MQLs[a] (LOQs)	$\%R_{app}$ (n=5)		%ME (n=5)		Repeatability %RSD (n=5)		Reproducibility %RSD (n=5)	
			Low[b]	High[c]	Low[b]	High[c]	Low[b]	High[c]	Low[b]	High[c]
Flephedrone	0.010	0.075	52	64	-18	-5	15	14	17	17
Ethcathinone	0.030	0.075	51	61	-19	-6	14	15	17	16
Buphedrone	0.030	0.075	53	62	-15	-6	14	15	16	17
2-MMC	0.015	0.075	50	61	-19	-6	17	14	14	16
Butylone	0.010	0.075	54	66	-21	-8	14	14	15	16
Mephedrone	0.010	0.075	60	65	-14	-8	20	14	17	16
4-MEC	0.020	0.075	52	61	-21	-8	14	15	16	16
Pentedrone	0.020	0.075	51	62	-18	-8	17	16	18	17
3,4-DMMC	0.010	0.075	55	66	-21	-7	19	14	15	14
Alpha-PVP	0.005	0.075	54	63	-17	-7	12	14	16	13
MDPV	0.003	0.075	53	64	-20	-10	13	13	14	11

[a] ng/g [b] 1 ng/g [c] 40 ng/g

Finally, to prove the accuracy of the method and because OF samples from drug abusers were unavailable, we used the method to analyse three blind samples spiked with cathinones by laboratory staff. Accuracy was expressed as a measure of the error between the concentration found in the sample and the concentration spiked by a laboratory staff member as explained in the method validation subsection in the experimental part. The errors were between 10 and 20% for the first sample spiked at a concentration of 0.45 ng/g, between 1 and 18%for the second sample spiked at 6.2 ng/g, and between 1 and 12% for the third sample spiked at 58 ng/g. These acceptable errors proved the method's reliability. These values are shown in Table 3 and a chromatogram obtained for the second blind sample (6.2 ng/g) is shown in Figure 4.

This method achieved lower MDLs and MQLs than other strategies with more laborious sample pre-treatment procedures that included an extraction step such as SPE, US-DLLME or MEPS (15, 18, 22, 23). Our fast, efficient and easy-to-handle sample pre-treatment method performed in the same collector device is able to achieve slightly lower (or even similar) recoveries than those obtained with other methods.

Table 3. % of error in the analyte concentration of the three blind OF samples analysed by LC-MS/MS

Analyte	Sample 1 (0.45 ng/g)	Sample 2 (6.2 ng/g)	Sample 3 (58 ng/g)
Flephedrone	19	11	3
Ethcathinone	18	4	6
Buphedrone	20	7	10
2-MMC	19	16	1
Butylone	10	18	2
Mephedrone	18	2	4
4-MEC	20	18	5
Pentedrone	12	1	9
3,4-DMMC	20	12	6
Alpha-PVP	18	1	12
MDPV	19	7	9

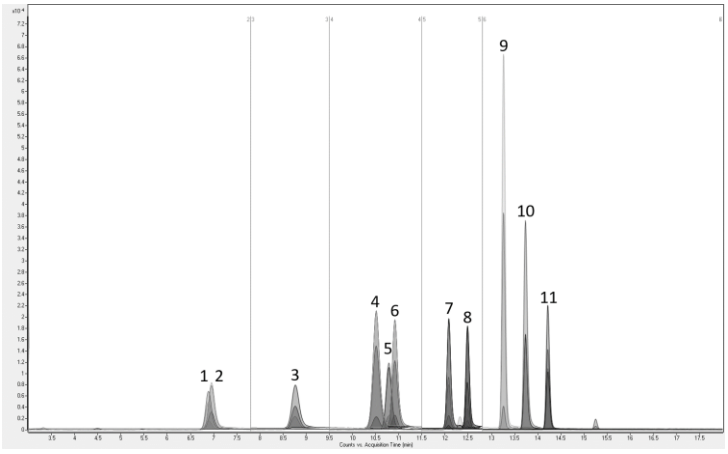

Figure 4. Chromatogram of a blind OF sample spiked with a mixture of the cathinones at 6.2 ng/g and analyzed by LC–MS-MS under the optimal conditions. Peak assignment: 1: flephedrone, 2: ethcathinone, 3: buphedrone, 4: 2-MMC, 5: butylone, 6: mephedrone, 7: 4-MEC, 8: pentedrone, 9: 3,4-DMMC, 10: alpha-PVP and 11: MDPV.

Conclusions

We have successfully developed and validated a method for determining synthetic cathinones in OF by LC-MS/MS using Salivette® as the collector device. The main novelty of our study is the simplicity of the sample pre-treatment. Our results show that this method can be used to quickly determine synthetic cathinones in OF samples with great sensitivity and without the need for laborious sample pre-treatment.

Moreover, the cathinones can be determined at the usual concentrations at which these compounds are present in OF from drug abusers. Finally, when the method was tested in spiked blind OF samples, it was shown to be a useful tool for forensic and toxicological applications.

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Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval

All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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DETERMINATION OF DRUGS OF ABUSE IN URINE AND ORAL FLUID SAMPLES
Sergi Pascual Caro

3.1.3. Determination of Synthetic Cathinones in Urine and Oral Fluid by Liquid Chromatography High-Resolution Mass Spectrometry and Low-Resolution Mass Spectrometry: A Method Comparison

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Abstract: Synthetic cathinones have become very popular recreational drugs. Therefore, determining them in biological samples is now a matter of concern. In recent years, different methods that have been developed can determine these drugs at low-concentration levels. In general, liquid chromatography mass spectrometry detection plays an important role in these methods and the trend is to use low-resolution and high-resolution mass spectrometry. In this article, for the first time, we compare these two analyzers using an Orbitrap and a triple quadrupole mass spectrometer in order to determine a group of synthetic cathinones in urine and oral fluid samples. For this comparison, we evaluated and compared different parameters: Method detection and quantification limits, linearity, apparent recoveries, matrix effect, repeatability (intra-day), reproducibility (inter-day), and accuracy. Similar results were obtained for the two analyzers for the apparent recoveries and matrix effect. However, triple quadrupole showed higher sensitivity compared to Orbitrap for both urine and oral fluid samples. The quantification limits in urine and the detection limits in saliva were two times lower for triple quadrupole. Finally, when blind samples were analyzed to study the accuracy, similar results were obtained for both analyzers.

Keywords: synthetic cathinones; solid-phase extraction; tandem mass spectrometry; high-resolution mass spectrometry; biological samples

1. Introduction

Synthetic cathinones are an important group among the new psychoactive substances (NPS) that have emerged in recent years as some of the most common drugs around the world [1,2]. These compounds are synthetized from the molecule cathinone, a natural alkaloid that can be found in the Catha edulis plant [3]. Although they were first synthetized for medical purposes, they began being used as recreational drugs and have emerged as an important alternative to other worldwide popular drugs such as cocaine, amphetamine, or ecstasy [3]. This is because they have similar effects to some of these drugs, such as paranoia and euphoria, which can be partly attributed to their similarity to amphetamine compounds [2,4]; however, cathinones have an additional ketone group at the β position of the amino chain. Moreover, when cathinones are combined with other drugs such as gamma-hydroxybutyric acid (GHB), gamma-butyrolactone (GBL), or cocaine they can reduce inhibition and enhance sexual activities [2,4]. Synthetic cathinones are sold as "bath salts" and can be found easily on different websites because drug agencies cannot control all of them due to their continuous structural changes [4,5].

When these substances are consumed, they can be present in different biological samples and they have become very important in toxicological and forensic analyses [6–20]. Although in these samples it is possible to find the parent drug and its metabolites, it is usually the latter that remain longer in the body. However, there are some matrices including blood and oral fluid (OF), in which the parent drug is not as metabolized as in others, such as in urine [21]. Moreover, depending on the biological sample, the detection window can be different and even this could be affected by several factors, such as the frequency and the amount of drug taken by the individual. In general, for short detection windows, breath, blood, or OF are the usual samples, for medium detection windows, urine and sweat are the most convenient, and for long detection windows, hair and meconium are the preferred choices [21–24]. In this work, we focused on urine and OF.

Although the concentration range of cathinones in urine and OF may depend on the amount of the drug consumed, in urine, low levels of these compounds in the range of ng mL^{-1} 24 h after the consumption are usual [25], while in OF, cathinones can be found between low and high levels of ng mL^{-1} due to its earlier detection [26]. Therefore, it is important to provide methods with high sensitivity to be able to detect the usual levels at which these substances can be found in biological samples from drug abusers. The instrumentation is, thus, a

key factor for achieving the goal of low detection limits. Mass spectrometry (MS) is a promising tool for this purpose. In the literature, there are several examples in which cathinones have been determined mainly using liquid chromatography (LC) due to the polar characteristics of these compounds. The trend is to use MS as a detection system to obtain methods with high sensitivity. Low-resolution MS, such as tandem MS (MS/MS), and high-resolution MS (HRMS) have become the preferred choices in recent years [6–9,11–20]. In the case of MS/MS, there are several examples using triple quadrupole (QqQ) or ion trap MS (QTrap) [6,7,11–16]. For HRMS, Orbitrap, QOrbitrap, and quadrupole time-of-flight (QToF) are the most used detectors [8,9,17–20]. Despite the satisfactory results obtained in these previously reported strategies, neither HRMS nor MS/MS have been compared for determining synthetic cathinones in biological samples as other compounds and matrices have been compared in the literature [27–35]. It has been proven that, in general, HRMS offers some advantages compared to MS/MS such as the target, post-target, and nontarget analyses, the possibility of operating in full-scan mode, and higher specificity. Thus, HRMS allows analyzing data for an unlimited number of compounds, with the possibility of performing targeted and untargeted analysis and to perform retrospective analysis. On the other hand, MS/MS normally offers better sensitivity and wider linear ranges. It also offers short dwell times to obtain very narrow peaks [27,34,36,37]. In particular, there are several examples in which QqQ and Orbitrap have been compared [27,28,30,32,33], and the authors concluded that both detectors can reach a similar sensitivity level. However, there are some examples that find significant differences between the compared MS analyzers. This was the case of Vanhaecke et al. [31], who reported higher sensitivity for the QqQ for determining anabolic steroids in meat, Gómez-Canela et al. [34], who reported higher sensitivity for the Orbitrap for characterizing multiclass cytostatic compounds, Kaufmann et al. [29], who obtained better sensitivity for the QqQ for determining veterinary drugs in honey and bovine kidney samples, and Viaene et al. [35], who achieved better sensitivity for the QToF for determining opioids in plasma. As it can be observed, the type of compound and the matrix in which they are determined are key factors when the sensitivity is evaluated.

Considering all the mentioned studies, the main objective of the present work was to establish, for the first time, an accurate comparison between MS/MS and HRMS in terms of sensitivity, accuracy, and other method validation parameters when synthetic cathinones are determined in urine and OF samples.

2. Materials and methods

2.1. Standards and materials

Eleven synthetic cathinones were selected for this study: 2-methyl-methcathinone (2-MMC), 3,4-dimethyl-methylcathinone (3,4-DMMC), 4-fluoro-methcathinone (flephedrone), 4-methylcathinone (4-MEC), 4-methyl-methcathinone (mephedrone), alpha-pyrrolidinovalerophenone (alpha-PVP), beta-ethylmethcathinone (pentedrone), buphedrone, butylone, methylene-dioxypyrovalerone (MDPV), and N-ethylcathinone. All of them were obtained from LGC Standards (Luckenwalde, Germany).

Different stock standard solutions for each cathinone were prepared in methanol (MeOH) at three concentrations: 100 mg L^{-1}, 1000 mg L^{-1}, and 2000 mg L^{-1}. A working standard mixture solution from the individual standard solutions was prepared in MeOH at 1 mg L^{-1} and then diluted in water to prepare the final solutions. All the standard solutions were stored in the freezer at −20 °C.

The solvents and reagents used in this study were: Acetonitrile (ACN) for LC-MS from Chem-Lab (Zedelgem, Belgium); water for LC-MS purchased from J.T. Baker (Deventer, The Netherlands); hydrochloric acid (HCl), formic acid (HCOOH), ammonium hydroxide (NH_4OH), sodium dihydrogen phosphate (NaH_2PO_4), and disodium hydrogen phosphate (Na_2HPO_4) from Sigma-Aldrich (St. Louis, MO, USA); and ultrapure water obtained using a water purification system (Merck Millipore, Darmstadt, Germany).

For urine, Oasis MCX (150 mg/6 cc) cartridges from Waters Corp. (Milford, MA, USA) were used. For OF, Salivette® devices were purchased from Starstedt (Nümbrecht, Germany) and a Hettich Universal 32R centrifuge machine from Hettich (Tuttlingen, Germany) was used to centrifugate the samples.

2.2. HPLC separation

The chromatographic conditions were optimized in a previous study in which the same group of cathinones were successfully separated [9].

A Luna Omega 5 µm Polar C18 (150 mm × 4.6 mm, 5 µm) with a Security Guard from Phenomenex (Torrance, CA, USA) was used for the chromatographic separation at 35 °C with a mobile phase flow rate of 0.6 mL min^{-1}. The sample injection volume was 20 µL and the vials were stored in the autosampler at 10 °C. The mobile phase was composed by A: 0.1% HCOOH in H2O and B: 0.1% HCOOH in ACN in gradient mode starting at 15% B, which was maintained for 5 min, increased to 35% in 5 min, then to 80% in 4 min, and to 100% in 1 min. Finally, it was maintained for 2 min before returning to the initial conditions in 1 min and held for 4 min.

2.3. Orbitrap

A Thermo Scientific Accela 1250 UHPLC system (Bremen, Germany) equipped with an Accela Autosampler automatic injector and an Accela 1250 pump coupled with a Thermo Scientific Exactive Orbitrap™ mass spectrometer were used. The instrument was also equipped with a higher-energy collisional dissociation (HCD) cell and a heated electrospray ionization (HESI) source.

The gas flow rate and temperature parameters were optimized with positive ionization and the following parameters were used: Auxiliary gas, 5 adimensional units (AU); sheath gas, 60 AU; capillary voltage, 30 volts (V); tube lens voltage, 80 V; spray voltage, 2 kV; and skimmer voltage, 24 V. The capillary and heater temperatures were set at 350 °C and 400 °C, respectively. The probe position was set at micrometer 1, side to side 0, and vertical B. Two-time windows were used for data acquisition in positive mode (0–11.75 min and 11.75–15 min). Two scan events in each window were set: Full scan at 50,000 Full Width at Half Maximum (FWHM) with 250 ms of injection time for the first one, and fragmentation scan at 10,000 FWHM with 50 ms of injection time for the second one. The HCD cell was set in "all ion fragmentation" mode with an injection time of 50 ms. The optimum collision voltages were 15 electronvolts (eV) for the first one and 25 eV for the second one. The mass ranges used for the full scan at high resolution were between 60 mass-to-charge ratio (m/z) and 300 m/z.

Table 1 specifies the LC-HRMS protonated molecules and fragments. The ion ratio was calculated by dividing the signal of the protonated molecule by the one of the fragment.

2.4. QqQ

The method was validated using an Agilent model 1200 series LC coupled with an Agilent 6460 series triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface from Agilent Technologies (Waldbronn, Germany).

The LC-MS/MS parameters were optimized by injecting individual standards at 1 mg L^{-1} into a mixture of H_2O:MeOH (50:50, volume/volume (v/v)). Five different windows were created according to their retention times: Flephedrone and ethcathinone; buphedrone; 2-MMC, butylone, and mephedrone; 4-MEC and pentedrone; and 3,4-DMMC, alpha-PVP, and MDPV. The optimized source parameters were: Gas flow rate, 13 L min^{-1}; gas temperature, 350 °C; nebulizer pressure, 60 psi; and capillary voltage 2500 V. The fragmentor was 100 V for most of the compounds and 125 V for 3,4-DMMC, alpha-PVP, and MDPV. The collision energies were between 8 and 25 eV. A multiple reaction monitoring (MRM)

mode was used, selecting the two most intensive transitions between the parent ion and the product ions. The optimized fragments are shown in Table 2. For the QqQ, the ion ratio was calculated by dividing the qualifier signal between the quantifier one.

2.5. Urine Collection and Preparation

To pretreat the urine samples, the same sample pretreatment procedure that was used in a previous study by our research group was applied [9]. Different urine samples from nonaddicted women and men volunteers of different ages were collected and mixed to obtain a pooled urine sample. All the volunteers consented to the study following the Declaration of Helsinki normative. The samples were collected in polypropylene tubes and kept in the freezer at −20 °C. Before their extraction, the samples were diluted with a phosphate buffer at pH 6 (0.15 molar (M) NaH_2PO_4 and 0.05 M Na_2HPO_4), 50:50 (v/v). The Oasis MCX cartridge was activated with 5 mL of MeOH, then conditioned with 5 mL of phosphate buffer solution (pH 6), and loaded with 5 mL of the mixture of urine:buffer 50:50 (v/v). After the loading, the cartridge was washed with 2 mL of MeOH and the analytes were eluted with 2 mL of 5% NH_4OH in MeOH. Finally, 100 µL of 1% HCl in MeOH were added prior to the evaporation to dryness under a gentle stream of N_2. Then, the obtained residue was reconstituted with 1 mL of mobile phase, filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter, and transferred to a vial for its chromatographic analysis.

2.6. Saliva Collection and Preparation

The saliva was pretreated using the same procedure as in a previous study by our research group [11]. Salivette® was used to collect the OF samples from nonaddicted volunteers of different ages following the Declaration of Helsinki normative. The cotton swab of the Salivette® was chewed and rolled around in the mouth for 1 min. This swab was returned to the tube and it was centrifuged for 1 min at 8000 rpm (10,800× g). The saliva fell to the bottom of the device and was discarded. Then 1 mL of MeOH was added to the swab and was centrifuged again at the same conditions. The centrifuge was collected, this procedure was repeated, and the two methanolic solutions were combined. Then, 100 µL of 1% HCl in MeOH were added to the solution before evaporating it to dryness under a gentle stream of nitrogen. The residue was then reconstituted with 500 µL of mobile phase under initial chromatographic conditions, filtered through a 0.45 µm PTFE filter, and the final solution was injected into the chromatograph.

2.7. Validation

The methods were validated following the guidance for the validation of analytical methodology and calibration equipment used for testing illicit drugs in seized materials and biological specimens and the European guidelines for workplace drug testing in oral fluid [38,39]. The developed methods were compared in terms of apparent recoveries (R_{app}), matrix effects (ME), method detection and quantification limits (MDLs and MQLs), linearity, and accuracy. The retention time in the analyte confirmation had to correspond to a tolerance of ±2.5% and the ion ratio to ±20%. Moreover, in the case of Orbitrap, the error mass had to correspond to a maximum tolerance of 5 ppm [40].

To evaluate the mentioned parameters, urine was spiked before the extraction procedure and OF was spiked in the cotton swab after it had been chewed. The $\%R_{app}$ in both cases was the recovery of all the methods, calculated from the ratio of the cathinone concentration of a sample spiked prior to the procedure and the concentration for the same cathinone in a standard sample. Both of these were obtained from the instrumental calibration curve.

Table 1. Retention time, protonated molecule (quantifier), the two fragment ions (qualifier), and the ratios of the studied cathinones by liquid chromatography high-resolution mass spectrometry (LC-HRMS).

		Protonated Molecule $[M+H]^+$		Fragment		
Analyte	**Rt (min)**	**Formula**	**Accurate Mass *m/z***	**Formula**	**Accurate Mass *m/z***	**Ratio**
Flephedrone	6.17	$C_{10}H_{13}FNO$	182.09757	$C_{10}H_{11}FN$	164.08755	38
				C_9H_8FN	149.06408	10
Ethcathinone	6.29	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	23
				$C_9H_{10}N$	132.08132	12
Buphedrone	7.66	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	19
				C_9H_9N	131.07350	6
2-MMC	8.97	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	30
				$C_{10}H_{11}N$	145.08915	16
Butylone	9.05	$C_{12}H_{16}NO_3$	222.11247	$C_{11}H_{12}NO$	174.09189	17
				$C_{12}H_{14}NO_2$	204.10245	17
Mephedrone	9.38	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	34
				$C_{10}H_{11}N$	145.08915	14
4-MEC	10.58	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	30
				$C_{10}H_{11}N$	145.08915	9
Pentedrone	10.98	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	16
				$C_9H_{10}N$	132.08132	10
3,4-DMMC	11.66	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	37
				$C_{11}H_{13}N$	159.10480	12
Alpha-PVP	12.21	$C_{15}H_{22}NO$	232.16959	C_7H_7	91.05478	15
				C_7H_5O	105.03404	5
MDPV	12.58	$C_{16}H_{22}NO_3$	276.15942	$C_8H_{16}N$	126.12827	8
				$C_8H_7O_2$	135.04461	9

Table 2. Retention time, fragmentor, precursor, product ions (the quantifier ion is underlined), ion ratios, and collision energies of the studied cathinones by LC-MS/MS.

Analyte	Rt (min)	Fragmentor (V)	Precursor ion	Product Ion	CE (eV)	Ion Ratio
Flephedrone	6.53	100	182.1	<u>164</u>	15	
				149	15	70
				123	15	21
Ethcathinone	6.62	100	178.1	<u>160</u>	12	
				132	14	93
				105	18	39
Buphedrone	8.29	100	178.1	<u>160</u>	10	
				131	15	55
				132	15	52
2-MMC	9.98	100	178.1	<u>160</u>	8	
				145	15	71
				119	15	11
Butylone	10.26	100	222.1	<u>174</u>	14	
				204	10	101
				191	10	23
Mephedrone	10.48	100	178.1	<u>160</u>	8	
				145	15	61
				119	15	12
4-MEC	11.81	100	192.1	<u>174</u>	10	
				146	16	21
				145	18	24
Pentedrone	12.25	100	192.1	<u>174</u>	10	
				132	16	49
				91	18	24
3,4-DMMC	13.02	125	192.1	<u>174</u>	10	
				159	15	57
				133	15	7
Alpha-PVP	13.50	125	232.2	<u>91</u>	22	
				105	25	42
				126	25	41
MDPV	13.96	125	276.2	<u>126</u>	25	
				135	25	67
				175	20	52

The percentage of ME was calculated according to the following Equation (1):

$$\%ME=[(C_{spiked}/C_{STD})\times100]-100 \quad (1)$$

C_{STD} is the concentration of a cathinone in a standard sample and C_{spiked} is the concentration of the same cathinone in urine or in an OF extract spiked at the same concentration as C_{STD} after the procedure.

In the case of Orbitrap, the MDLs were defined as the lowest detectable concentrations with a signal intensity higher than $\times10^3$ for the protonated molecule and for the fragments, while the MQLs were considered the lowest point in the calibration curve. However, for the QqQ, the MDLs were the minimum concentrations with a signal to noise (S/N) ratio greater than 3 and the MQLs were the lowest point in the

calibration curve with a S/N ratio higher than 10.

The method linearities were based on a matrix-matched calibration curve by evaluating the determination coefficient (r^2). Two concentration levels were used to study the Rapp and ME: 1 ng g^{-1} (low) and 40 ng g^{-1} (high) for OF and 1 ng mL^{-1} (low) and 40 ng mL^{-1} (high) for urine. In the case of OF, the concentrations were expressed as nanograms (ng) of cathinone per gram of OF weighted (ng g^{-1}), and ng was used for the amount of the cathinone in OF in the calibration curve. In the pretreatment, the Salivette® was weighed before and after the sampling to determine the amount of saliva sampled. Thus, to express the concentration of a cathinone in a sample, the ng found was divided by the grams of saliva weighed.

Finally, to determine the accuracy of the methods, we analyzed different blind samples spiked with all the cathinones by a laboratory staff member at different concentrations. The concentration found in these samples was calculated, compared to the spiked value, and the accuracy in terms of error was calculated for each compound.

3. Results and discussion

In order to evaluate both LC-MS/MS and LC-HRMS methodologies, the methods for urine and OF samples for the two detectors were validated to compare their performance in terms of linearity, MDLs, MQLs, R_{app}, ME, and accuracy of the methods.

3.1. Urine Samples

3.1.1. Method Recoveries and Matrix Effects

As it was mentioned above, $\%R_{app}$ was evaluated at low and high-concentration levels (1 ng mL^{-1} and 40 ng mL^{-1}). For the low concentration tested, the values of $\%R_{app}$ for the different cathinones ranged from 73 to 125% for Orbitrap and from 53 to 98% for QqQ, while for the higher concentration value the recovery values ranged between 69 and 88% and 72 and 87%, respectively (Figure 1).

In the case of ME (Figure 2), for Orbitrap at 1 ng mL^{-1}, suppression values were between −8 and −28% for most of the compounds. Enhancements of 12 and 21% were obtained for 4-MEC and alpha-PVP, respectively. At 40 ng mL^{-1}, suppressions between −9 and −22% were obtained for all the compounds. However, for QqQ at the low-concentration level (1 ng mL^{-1}), values from −14 to −33% were obtained for most of the compounds except for flephedrone and 3,4-DMMC, which had enhancements of 12 and 10%, respectively. At the high level (40 ng mL^{-1}), ME values ranged between −5 and 20% for QqQ.

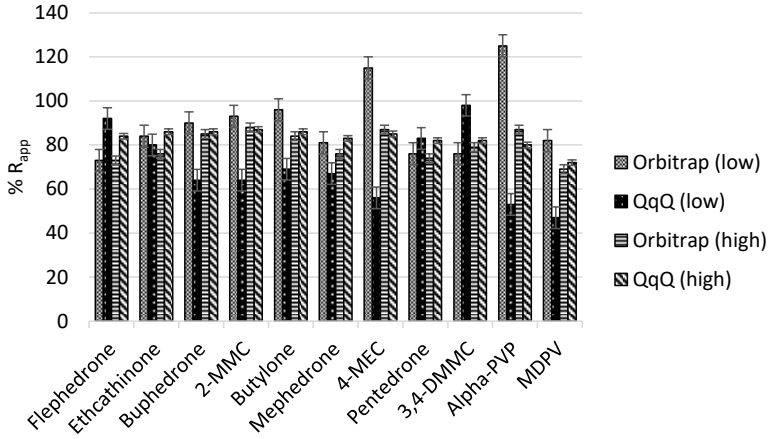

Figure 1. The percentage of apparent recovery (%R_{app}) at low (1 ng mL^{-1}) and high (40 ng mL^{-1}) levels of concentrations for Orbitrap and triple quadrupole (QqQ) in the analysis of synthetic cathinones in urine.

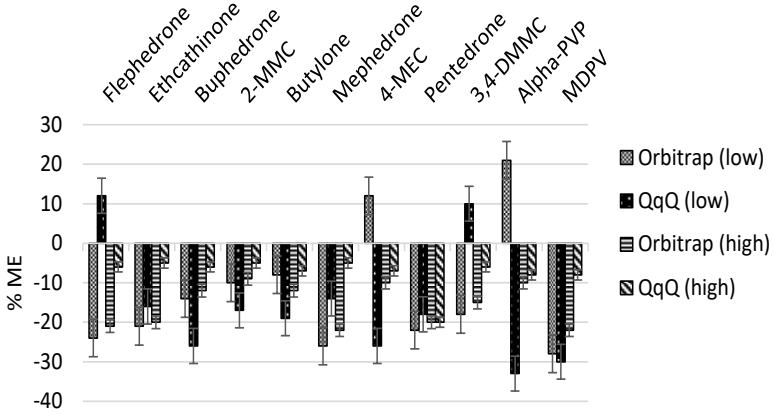

Figure 2. The percentage of matrix effect (%ME) at low (1 ng mL^{-1}) and high (40 ng mL^{-1}) levels of concentrations for Orbitrap and QqQ in the analysis of synthetic cathinones in urine.

In order to statistically compare both instruments in terms of %R_{app}, a two-way ANOVA was performed. At the low-concentration level, LC-HRMS achieved slightly better results compared to LC-QqQ. The results of the statistical test proved that there were significant differences between both MS systems (critical Fisher (F)-value: 4.96; F-obtained: 7.85). On the contrary, at the high concentration, no significant differences between both instruments were found (critical F-value: 4.96; F-obtained: 2.88).

3.1.2. Linearity, MDLs, and MQLs

Matrix-matched calibration curves were used for quantification in the two methods to compensate the ME and the possible losses in the sample pretreatment. Table 3 shows the linearity range and the MDLs and MQLs obtained when the two MS analyzers were used to analyze cathinones in urine. In the case of Orbitrap, the linear range was from 0.050 to 4 ng mL^{-1} and from 4 to 200 ng mL^{-1} for most of the cathinones and from 0.200 to 4 ng mL^{-1} and from 4 to 200 ng mL^{-1} for ethcathinone, buphedrone, and 4-MEC. For QqQ, the linear range was between the MQL and 4 ng mL^{-1} and from 4 to 200 ng mL^{-1}. The linear ranges for the two analyzers encompassed the usual concentrations at which these compounds can be found in urine samples from drug abusers. The MQLs for QqQ ranged from 0.020 to 0.070 ng mL^{-1} and for Orbitrap they were 0.050 ng mL^{-1} for most analytes and 0.200 ng mL^{-1} for ethcathinone, buphedrone, and 4-MEC. The MDLs were between 0.005 and 0.035 ng mL^{-1} for Orbitrap and between 0.040 and 0.160 ng mL^{-1} for QqQ.

Comparing all these values, it was possible to conclude that with QqQ lower MDLs and MQLs were obtained and, therefore, the method based on LC-MS/MS has better sensitivity for the analyzed cathinones in urine samples than the one based on HRMS. In different studies reported in the literature focused on the comparison of different MS analyzers, the general conclusion was that no significant differences between LC-HRMS and LC-MS/MS in terms of sensitivity were observed [27–35]. However, there are some examples in which the results are coincident with those obtained in the present study, as in the case of Vanhaecke et al. [31] and Kaufmann et al. [29], who also obtained better sensitivity for QqQ, although it is important to point out that these authors analyzed different compounds and the matrices were also different to the ones analyzed in this study.

3.1.3. Accuracy

To study the accuracy of the method, three different blind urine samples (A, B, and C) spiked by a laboratory staff member were analyzed using the two methods, LC-Orbitrap and LC-MS/MS. The three samples were spiked at different concentrations: 2 ng mL^{-1} (A), 60 ng mL^{-1} (B), and 125 ng mL^{-1} (C). The accuracy was calculated in terms of percentage of error between the concentration obtained for each cathinone by analyzing these blind samples and the spiked concentration for each analyte. Finally, the average percentage of error was calculated for each sample. In the case of Orbitrap, values of 18% (A), 6% (B), and 15% (C) were obtained and for QqQ the values were 16% (A), 1% (B), and 15% (C).

Table 3. Linearity, method detection limits (MDLs) and method quantification limits (MQLs) for Orbitrap and QqQ in ng mL^{-1} for the determination of synthetic cathinones in urine.

	MDL Orbitrap	MDL QqQ	MQL Orbitrap	MQL QqQ	Linear Range Orbitrap	Linear Range QqQ
Flephedrone	0.080	0.025	0.200	0.050	0.200 – 4 4 - 200	0.050 – 4 4 - 200
Ethcathinone	0.100	0.030	0.050	0.050	0.050 – 4 4 - 200	0.050 – 4 4 - 200
Buphedrone	0.100	0.035	0.200	0.050	0.200 – 4 4 - 200	0.050 – 4 4 - 200
2-MMC	0.100	0.025	0.050	0.050	0.050 – 4 4 - 200	0.050 – 4 4 - 200
Butylone	0.120	0.030	0.050	0.050	0.050 – 4 4 - 200	0.050 – 4 4 - 200
Mephedrone	0.080	0.005	0.050	0.030	0.050 – 4 4 - 200	0.030 – 4 4 - 200
4-MEC	0.040	0.010	0.200	0.030	0.200 – 4 4 - 200	0.030 – 4 4 - 200
Pentedrone	0.040	0.025	0.050	0.030	0.050 – 4 4 - 200	0.030 – 4 4 - 200
3,4-DMMC	0.040	0.010	0.050	0.030	0.050 – 4 4 - 200	0.030 – 4 4 - 200
Alpha-PVP	0.160	0.005	0.050	0.020	0.050 – 4 4 - 200	0.020 – 4 4 - 200
MDPV	0.060	0.005	0.050	0.030	0.050 – 4 4 - 200	0.030 – 4 4 - 200

According to the validation guide, the results have to fall within ±20% at the lower concentration and ±15% at the higher concentration [36]. From these results it was possible to observe that all the values obtained were within the range mentioned and, therefore, they were considered very good results for the method accuracy. Moreover, no significant differences between LC-HRMS and LC-MS/MS were observed in terms of accuracy.

3.2. OF Samples

3.2.1. Method Recoveries and Matrix Effects

The $\%R_{app}$ and ME for the OF samples were also evaluated, as for the urine samples, at the same two concentration levels: 1 ng g^{-1} and 40 ng g^{-1}. For both MS analyzers higher $\%R_{app}$ was obtained at the high-concentration level evaluated and higher ME were obtained at the low-concentration level. R_{app} at the low level ranged from 41 to 53% for Orbitrap and 50 to 60% for QqQ, while at high levels it ranged from 50 to 55% and from 61 to 66%, respectively (Figure 3). The ME values observed at the low level were between −7 and −20% for Orbitrap and between −14 and −21% for QqQ. ME at the high level ranged from −1 to −9% for Orbitrap and from −5 to −10% for QqQ (Figure 4).

In the same case as urine, a two-way ANOVA was used to compare both detectors in OF. At both levels of concentration, significant differences could be observed, obtaining better results for the LC-QqQ (critical F-value: 4.96; F-obtained: 5.98 at low concentration and critical F-value: 4.96; F-obtained: 265.38 at high concentration).

3.2.2. Linearity, MDLs, and MQLs

Similarly to the urine samples, a matrix-matched calibration curve for the OF samples was used. Table 4 shows the linearity range and MDL and MQL parameters studied for analyzing

cathinones in OF. Similar linear ranges were obtained for Orbitrap and QqQ. For QqQ, the calibration curves were adjusted to two linear ranges, from 0.075 to 4 ng and from 4 to 75 ng of cathinone in OF for all the compounds. The Orbitrap calibration curves were also adjusted to the two same linear ranges for most of the compounds except for buphedrone and 4-MEC, which were from 0.100 to 4 ng and from 4 to 75 ng of cathinone in OF. These linear ranges covered all the usual concentrations at which cathinones can be found in OF from drug abusers.

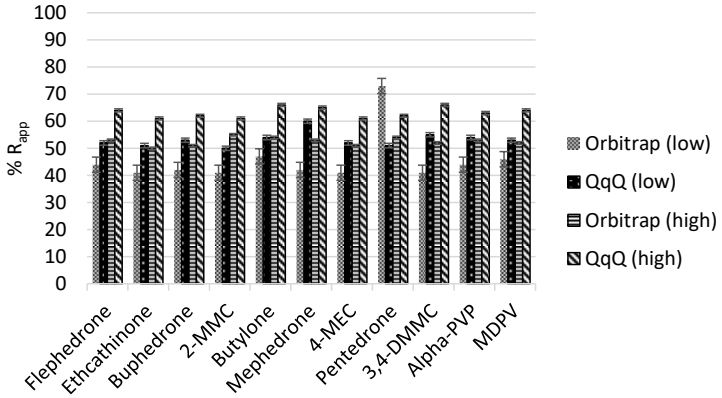

Figure 3. The $\%R_{app}$ at low (1 ng g^{-1}) and high (40 ng g^{-1}) levels of concentrations for Orbitrap and QqQ in the analysis of synthetic cathinones in oral fluid (OF).

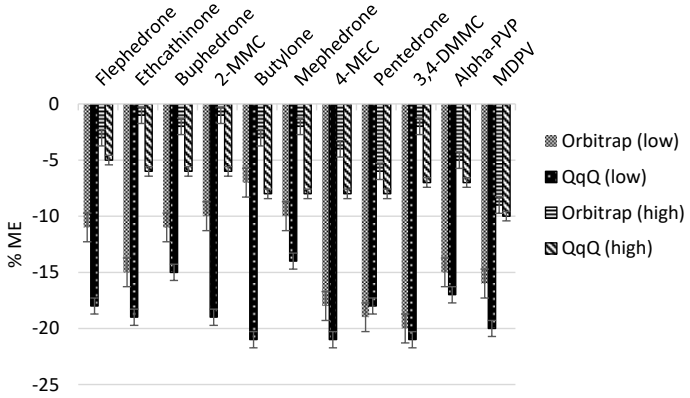

Figure 4. The %ME at low (1 ng g^{-1}) and high (40 ng g^{-1}) levels of concentrations for Orbitrap and QqQ in the analysis of synthetic cathinones in OF.

Table 4. Linearity in ng of cathinones, MDLs, and MQLs in ng g^{-1} for Orbitrap and QqQ for the determination of synthetic cathinones in OF.

	MDL Orbitrap	MDL QqQ	MQL Orbitrap	MQL QqQ	Linear Range Orbitrap	Linear Range QqQ
Flephedrone	0.020	0.010	0.075	0.075	0.075- 4 4 - 75	0.075 – 4 4 – 75
Ethcathinone	0.035	0.030	0.075	0.075	0.075- 4 4 – 75	0.075 – 4 4 – 75
Buphedrone	0.025	0.030	0.100	0.075	0.100 - 4 4 – 75	0.075 – 4 4 – 75
2-MMC	0.015	0.015	0.075	0.075	0.075- 4 4 – 75	0.075 – 4 4 – 75
Butylone	0.020	0.010	0.075	0.075	0.075- 4 4 – 75	0.075 – 4 4 – 75
Mephedrone	0.010	0.010	0.075	0.075	0.075- 4 4 – 75	0.075 – 4 4 – 75
4-MEC	0.025	0.020	0.100	0.075	0.100- 4 4 – 75	0.075 – 4 4 – 75
Pentedrone	0.020	0.020	0.075	0.075	0.075- 4 4 – 75	0.075 – 4 4 – 75
3,4-DMMC	0.015	0.010	0.075	0.075	0.075- 4 4 – 75	0.075 – 4 4 – 75
Alpha-PVP	0.010	0.005	0.075	0.075	0.075- 4 4 – 75	0.075 – 4 4 – 75
MDPV	0.015	0.003	0.075	0.075	0.075- 4 4 - 75	0.075 – 4 4 – 75

Thus, the MQL, which was considered as the lowest point in the calibration curve, was 0.075 ng g^{-1} for all the compounds in the case of QqQ. For Orbitrap, they were 0.075 ng g^{-1} for most of the compounds except for buphedrone and 4-MEC, for which they were 0.100 ng g^{-1}. The MDLs were slightly lower in the case of QqQ, between 0.003 and 0.030 ng g^{-1}, and they were between 0.010 and 0.035 ng g^{-1} in the case of Orbitrap.

Comparing both the MDLs and MQLs for the two analyzers, lower values were obtained, in general, when LC-MS/MS was used. Therefore, better sensitivity was obtained with this analyzer, despite only slight differences.

3.2.3. Accuracy

To study the accuracy of the method for OF samples, three different blind samples spiked by a member of the laboratory at different concentrations (0.45 ng g^{-1} (A), 6.2 ng g^{-1} (B), and 58 ng g^{-1} (C)) were analyzed with the two methods. The % errors obtained for Orbitrap were 16% (A), 10% (B), and 15% (C), while for QqQ the % errors were 18% (A), 9% (B), and 6% (C). These values were within the acceptable limits according to the guide and are considered excellent results in both cases [36]. The results show that there were no observable differences and in both cases good results were achieved.

4. Conclusions

Two analytical methods for determining synthetic cathinones in urine and OF by LC-HRMS and LC-MS/MS were compared in terms of linearity, MDLs, MQLS, %Rapp, %ME, and accuracy. For both biological samples, better sensitivity was observed in the case of LC-MS/MS and no significant differences were found when $\%R_{app}$ and %ME were compared. Finally,

the two methodologies demonstrated good accuracy values and, although similar values were obtained for urine and OF samples, the latter showed slightly better results for LC-MS/MS. For these reasons, in general terms, we can say that LC-MS/MS can provide better sensitivity and accuracy when synthetic cathinones are determined in urine and OF. Nevertheless, it was also observed that the two methodologies (based on either HRMS or MS/MS) both have good potential for forensic and toxicological analyses. With these methods, low concentrations of these substances can be found in urine and OF samples, which is interesting from the juridical point of view. The developed research can be considered an important starting point to apply the different methods for urine and OF samples in monitoring campaigns, which can be focused in the study of the trends of the cathinone consumption in different regions, and to establish possible relationships between the consumption and the age of the consumers.

Author Contributions

S.P.-C.: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing-original draft preparation, writing-review and editing, visualization.

F.B.: conceptualization, resources, project administration, funding acquisition.

C.A.: conceptualization, validation, formal analysis, investigation, resources, data curation, writing-review and editing, visualization, supervision, project administration, funding acquisition.

M.C.: conceptualization, validation, formal analysis, investigation, resources, data curation, writing-review and editing, visualization, supervision, project administration, funding acquisition.

All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

OF, oral fluid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HRMS, high-resolution mass spectrometry; QqQ, triple quadrupole; R_{app}, apparent recovery; ME, matrix effect; MDL, method detection limits; MQL, method quantification limits.

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3.1.4. Discussion of results

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Although the results of the experimental part of the studies included in this section have been already discussed in their respective papers, the most relevant aspects of these results are summarized and discussed below. These studies were based on the development of sensitive methodologies for the determination of a group of eleven synthetic cathinones in urine and OF. Moreover, a comparison of the two instrumentations used in each study, LC-HRMS and LC-MS/MS have been carried out for both matrices.

Urine is a widely employed matrix in toxicological analyses but it can contain compounds that may interfere with the analytes of interest [1,2]. For that reason, a SPE has been carried out to clean, extract and preconcentrate eleven cathinones. In particular, two commercial mixed-mode cation-exchange sorbents (Oasis WCX and Oasis MCX) were firstly evaluated and compared for the SPE procedure. These two sorbents are capable of retaining basic analytes through cation-exchange and reversed-phase interactions. Even that the extraction efficiencies of both sorbents were similar (around 90% for most of them), when the clean-up step was studied, Oasis WCX obtained worse results. In the optimisation of the clean-up step for Oasis WCX, MeOH and different proportions of MeOH:H_2O were evaluated but all of them eluted the analytes and thus, H_2O was chosen as washing solvent. For Oasis MCX, MeOH was suitable to remove the interferences without eluting the analytes. Moreover, a study based on the differences between performing or not the clean-up step for the two sorbents was carried out. It was observed that the ME of both sorbents were decreased when the washing step was performed. However, the %ME obtained with Oasis MCX were lower than with Oasis WCX, maybe because the first used MeOH as washing solvent and the last used H_2O, which was not capable to remove such interferences. As Oasis MCX achieved lower %ME than Oasis WCX, the $\%R_{app}$ of this sorbent were also studied, which were between 69 and 115%. The MDLs and MQLs were also studied, showing that the developed method could detect and quantify lower concentrations than some methods previously reported in which other SPE sorbents or other strategies were used [3–5].

OF has become a perfect alternative to blood as it overcomes some of its difficulties of collection and invasiveness, and offering the same window of detection [6–8]. The objective of the second study was to simplify the pre-treatment to achieve an easy and fast methodology collecting the sample with a

Salivette® device. The optimised procedure consisted on placing the swab in the mouth and chewing for one minute, as it was indicated for the manufacturer [9]. Then, after returning it to the tube it was centrifuged and the solution dropped at the bottom of the tube was discarded. MeOH was added to the swab and a centrifugation was performed to collect the solution (this was repeated twice and the solutions were mixed). Moreover, another parameter studied was the collection of the extract of the first centrifugation, that is the extract of the pooled saliva at the bottom of the collector. Several authors kept this extract and perform a further pre-treatment with it [10–13]. In our case, we studied the effects of keeping or discarding this first extract of the centrifugation. For this, the %ME and the $\%R_{app}$ were evaluated. Similar $\%R_{app}$ were obtained in both cases, which meant that the first centrifugation did not elute the analytes, or only a part of them. In the case of %ME, it was observed that if the first extract was discarded, lower values were obtained (13% less). Therefore, by discarding the first extract, similar $\%R_{app}$ and lower %ME were obtained, achieving a cleaner extract with less interferences. Moreover, it is important to remark that even that not and additional pre-treatment was performed to preconcentrate the analytes, very low levels of ng mL^{-1} were reached, lower than some other strategies performing SPE or LLE [10,14,15].

Even that a preconcentration and a clean-up step was carried out in urine samples while in OF the analytes were only desorbed from the swab, the methodology developed with OF achieved similar %ME than in urine and similar MDLs and MQLs. However, the $\%R_{app}$ obtained with the SPE in urine were higher than the ones obtained with the rapid pre-treatment in OF. As these two methodologies were developed with different instrumentation (LC-HRMS and LC-MS/MS), a comparison between these two MS detectors for determining a group of cathinones in urine and OF was performed. For that, each methodology was validated for each MS detector and the %ME, $\%R_{app}$, MDLs and MQLs obtained with both of them were compared. In the case of %ME, it was observed that LC-HRMS provided better results at lower concentrations than LC-MS/MS. Regarding the $\%R_{app}$, higher values were achieved using the LC-HRMS for urine at low concentration levels, while better results at the same levels were obtained for the LC-MS/MS in OF. In terms of MDLs and MQLs, for both urine and OF samples, the best results were obtained with LC-MS/MS, proving its higher sensitivity

compared to LC-HRMS. Therefore, even that both methodologies developed for determining synthetic cathinones in urine and OF using LC-HRMS and LC-MS/MS are suitable for toxicological and forensic analyses, the LC-MS/MS can reach lower concentration levels than the LC-HRMS.

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3.2. Determination of amphetamine-type substances in urine

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ATS is one of the most consumed types DOAs around the world and it is constituted by different substances in which AMP, MAMP and MDMA are the most popular. They present a chiral centre in the molecule and thus, two enantiomeric forms with different pharmacokinetic and pharmacological properties. Therefore, both enantiomers can be used for different purposes. It is the case of AMP, which is present in ADHD pharmaceuticals as S-enantiomer, while the R form is responsible of the stimulant effects. On the contrary, S-MAMP produces the effects related to drug use while the R-enantiomer is present in nasal decongestant products. Even though, in most drugs, both enantiomers are present as they are difficult to isolate but in the pharmaceuticals, the % of the enantiomer responsible of the stimulant effects is controlled [1–4].

These substances have been determined by several authors in urine samples from drug users and from individuals with medical prescription of pharmaceuticals containing ATS. They have used LC and GC, being LC the most common technique, and in most cases, coupled to MS/MS [5–7]. As urine is known to be a matrix that can contain some compounds that may interfere with the analytes of interest, a pre-treatment step is usually necessary. The sample pre-treatment performed in the present work was based on a previous published study of our group in which two mixed-mode cationic exchange sorbents (Oasis WCX and Oasis MCX) were evaluated for a group of synthetic cathinones in urine [8]. So, considering the similar characteristics between cathinones and ATS, as they only differ in the ketone group present in the cathinones, the same sorbents were studied for AMP, MAMP, MDMA and MDA. These analytes were selected because AMP, MAMP and MDMA are the most consumed ATS and because AMP is the metabolite of MAMP and MDA the metabolite of MDMA [9].

It is important to distinguish the two enantiomers of some ATS, as they can be taken for medical treatments or for drug abuse. So, without their enantioseparation, it is not possible to differentiate them and thus, positive samples in ATS would come from patients under medical prescription. As CE allows the easy enantioseparation of chiral compounds by the addition of a chiral selector in the background electrolyte (BGE) and based on the experience of our research group working with CE and more precisely, in the enantiomeric separation of DOAs such as synthetic cathinones, this section is focused on the enantiomeric separation of four ATS using CE coupled to MS/MS. Chiral

separation using CE is widely used, but when it is coupled to a MS system, it is important to use volatile and low conductivity BGEs and chiral selectors compatible with the MS to avoid sensitivity problems caused by source dirtying [10–12].

Despite that some authors have coupled CE to MS, the strategies developed need to prevent the entrance of incompatible chiral selectors into the MS and thus, compatible selectors and volatile and low conductivity BGEs have to be used. CDs are the most used chiral selectors in CE and different charged CDs have been modified for their compatibility with MS. Apart from the use of compatible chiral selectors, different strategies can be used to minimise the entrance of these substances to the MS such as the counter-current migration technique (CMT) or the partial filling technique (PFT) [11–14]. In the present study, due to the availability of different type of chiral selectors, some modified CDs and other chiral selectors have been evaluated using the CMT.

Therefore, the aim of this study is the development of a method for the enantiomeric determination of four ATS in urine by CE-MS/MS. In this sense, an evaluation and comparison of different chiral selectors and compatible strategies with the CE-MS/MS to achieve the best enantioseparation have been performed. This study has been published in the scientific journal Electrophoresis (2021) 43,437–445 and it is presented in section 3.2.1. in scientific article format.

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3.2.1. Comparison of different chiral selectors for the enantiomeric determination of amphetamine-type substances in human urine by solid-phase extraction followed by capillary electrophoresis-tandem mass spectrometry

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Comparison of different chiral selectors for the enantiomeric determination of amphetamine-type substances in human urine by solid-phase extraction followed by capillary electrophoresis-tandem mass spectrometry

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Abstract

The present study develops a method for the enantioseparation of a group of amphetamines and their metabolites in urine by CE coupled to MS/MS (CE-MS/MS). Amphetamines present a chiral center and thus two enantiomers, which is important from a toxicological point of view because they may have different pharmacokinetic and pharmacological properties. It is therefore essential to find suitable methods to distinguish both enantiomers. Today the use of CE is becoming more important in this field since, with the simple addition of a chiral selector to the background electrolyte, the enantioseparation can easily be achieved. However, when CE is coupled to MS, the use of volatile chiral selectors and compatible background electrolytes or other strategies such as the counter-current migration approach are required to avoid contamination of the ion source from non-volatile species. In the present study we use the latter strategy to evaluate six different chiral selectors using CE-MS/MS. As a sample pre-treatment, two cationic-exchange sorbents – Oasis WCX and Oasis MCX – are compared for the urine pre-treatment. Using this method, it was possible to achieve the complete chiral separation of the amphetamines under study with detection limits ranging between 0.8 and 1.5 ng/mL and method quantification limits between 2.0 and 8.0 ng/mL. Matrix-matched calibration curves up to 150 ng/mL were used to cover the usual concentration ranges at which amphetamines have generally been found in toxicological and forensic analyses.

Keywords: *Amphetamine-type substances · Cation-exchange sorbents · CE-MS/MS · Chiral selectors · Human urine*

Abbreviation*:* ***AMP****, amphetamine;* ***ATS****, amphetamine-type substances;* ***CMT****, countercurrent migration technique;* ***IDL****, instrumental detection limit;* ***IQL****, instrumental quantification limit;* ***MAMP****, methamphetamine;* ***MDL****, method detection limit;* ***ME****, matrix effect;* ***MeOH****, methanol;* ***MQL****, method quantification limit;* ***SFC****, supercritical fluid chromatography*

1. Introduction

The World Drug Report of 2020 stated that 0.5% of the adult European population had taken amphetamine-type stimulants (ATS) the previous year, this being the second most-consumed group of drugs. It also highlighted that consumption is increasing year by year [1,2]. ATS include some of the most popular drugs in the illegal market, such as amphetamine (AMP), meth-amphetamine (MAMP) and 3,4-methylenedioxymethamphetamine (MDMA or ecstasy) [2,3]. These drugs have an asymmetric carbon in their molecule and so they may occur in the form of R and S enantiomers with different pharmacokinetic, pharma-cological and toxicological properties [4]. In some cases, as both enantiomers may produce different effects, their control and legality are different. This is why their proper identification in the forensic field is so important [4]. In general, the S enantiomers have greater potency than the R enantiomers [5]. For example, although AMP is widely known as an illegal drug, it also has a medical application. The prescribed AMP is S-AMP or its prodrug Lisdexamphetamine, which is used to treat adults with attention-deficit hyperactivity disorder (ADHD) [6,7]. Regarding R-AMP, it was first taken in the World War II to promote wakefulness and in the 1950s, it was stablished as abuse drug in the illegal market [4]. In the case of MAMP, the R-enantiomer is known to be a nasal decongestant in Vicks VapoInhaler devices [8]. In the synthesis of pharmaceuticals, the aim is to obtain the single active enantiomer responsible for the therapeutic properties, but many of them are marketed as racemates, with still small percentages of the other enantiomer. In the case of illicit drugs, racemates are normally found together with other additives because clandestine laboratories do not usually isolate the enantiomer that functions as a central nervous system stimulant for economic reasons mainly because of the production price and because the consumption of the single pure enantiomer could lead to overdose or dead in some cases. Therefore, to differentiate between an ATS from a medical prescription consumption and from a drug abuse, it is important to be able to isolate both enantiomers in toxicological and forensic analyses [5].

Once ATS are taken they are metabolized in the body, and consequently these compounds and their metabolites can be found in different biological matrices. Thus, when biological samples are analysed, apart from MAMP, their main metabolites p-hydroxymethamphetamine (OHMA) and AMP can be found. Similarly, MDMA can also give rise to their metabolites 4-hydroxy-3-methoxy- methampheta-mine (HMMA) and 3,4-methylenedioxy-amphetamine (MDA) [9,10]. Therefore, it is important to be aware that AMP can

be found due to drug intake and also due to the metabolization of MAMP. In the present study AMP, MAMP, MDMA and MDA are the compounds chosen for studying both parent compounds and some of their main metabolites. Not all the different biological samples used to detect drug consumption present the same detection times (detection window) and, depending on the aim of the intended study, it might be better to analyse one matrix rather than another. Urine is one of the most frequently used biological samples and has a wide detection window stretching from days to weeks. In this biological sample ATS can be found ranging from low levels of ng/mL to values higher than 1000 ng/mL [9,11–15]. The main advantages of urine are that huge quantities of sample are easily obtainable, no trained personnel are needed to collect it, and the parent drugs and their metabolites can be found in higher concentrations compared to other matrices [16–18].

The separation of enantiomers can be easily accomplished by capillary electrophoresis (CE) [19–24], although supercritical fluid chromatography (SFC) [11], gas chromatography (GC) [12,25–27] and liquid chromatography (LC) [13,14,28] have also been used by some authors. The main advantages of CE are its high separation efficiency and the fact that it is an environmentally-friendly technique. Its main drawback is its limited sample loading capacity, which means reduced sensitivity, especially when used in combination with ultraviolet detection [29–31]. The advantages of CE in chiral analyses are related to the rapid method development and its flexibility when it comes to choosing or changing the chiral selector and its concentration, since the enantioseparation is performed simply by adding this substance to the background electrolyte (BGE) [11,30,32–34]. However, when a chiral CE method is combined with MS it is important to take into account that, apart from volatile and low conductivity BGEs, compatible chiral selectors to prevent source dirtying should be used since this can cause problems when low sensitivity is required [29,30,34,35]. Although CDs are the most common chiral selectors used in CE, they are non-volatile compounds and may bring about source contamination of the MS system. This can result in the ion suppression of the MS signal and consequently the sensitivity of the MS will be lower [30,32,34,36]. Although lowering the concentration of the chiral selector can minimize these drawbacks, different strategies have been reported in the literature as regards minimizing the contamination of the ionization source [34]. One of these is the countercurrent migration technique (CMT), in which the selected chiral selector migrates in the opposite direction to the electroosmotic flow (EOF) [35,36]. To achieve this, charged

chiral selectors are required. Since ATS are basic compounds with pK_a values of around 10, at a lower pH than their pK_a they are positively charged. When using CMT, therefore, negatively charged chiral selectors such as sulphated-CD, phosphated-CD, sulphobutylated-CD, macrocyclic antibiotics or crown ethers are needed. This approach has been used in some examples previously reported in the literature that have focused on the chiral determination of ATS by CE-MS [21–24]. Charged CDs were used in all of them, with sulphated-CD being the most usual.

Urine is a complex sample and the matrix components may interfere in the determination of the analytes of interest. A pre-treatment should therefore be carried out when this matrix is selected for ATS determination. The most common extraction techniques used by authors for this purpose are liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [11,13,14,19,20,25–27].

The main aim of this study is to develop a method for the enantio-determination of a group of ATS and their metabolites in urine samples by SPE CE-MS/MS. For the enantiose-paration of the selected ATS, six different chiral selectors are evaluated and compared using CMT in order to be able to couple the method with MS detection. As far as we know, this is the first study in which different chiral selectors are compared for the enantio-separation of ATS in urine by CE-MS/MS. Five of these chiral selectors are based on charged CDs, while the last is a macrocyclic antibiotic. For the urine pre-treatment, two cationic exchange sorbents – Oasis WCX (weak) and Oasis MCX (strong) – are also compared to extract the ATS from urine.

2. Materials and methods

2.1. Standards and materials

The analytical standards of AMP, R-AMP, S-AMP, MAMP, R- MAMP, S-MAMP, MDA and MDMA were purchased from Sigma Aldrich (St. Louis, MO, United States). The chiral selectors α-CD sulphated sodium salt, β-CD sulphated sodium salt, γ-CD sulphated sodium salt, γ-CD phosphate sodium salt and β-CD sulphobutylated sodium salt were obtained from Cyclolab (Budapest, Hungary). Vancomycin was obtained from LGC Standards (Teddington, UK).

Standard solutions of the ATS were prepared individually in MeOH at 100 mg/L and kept in the freezer at -20 °C. The working solutions were prepared by diluting a mixture solution containing the ATS at a concentration of 1 mg/L in MeOH with water at different con-centrations. These solutions were also kept in the freezer at -20 °C.

Ultra-gradient HPLC grade methanol (MeOH) and 2-propanol (IPA) were

obtained from J.T. Baker (Deventer, The Netherlands). Formic acid (HCOOH), ammonium acetate, ammonium formate and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich. Methanol for LC-MS was obtained from Chem-Lab (Zedelgem, Belgium). Water and acetonitrile (ACN) for LC-MS were purchased from Scharlab (Barcelona, Spain). Ultrapure water was obtained using a water purification system (Meck Millipore, Darmstadt, Germany).

The capillary used was purchased from Polymicro Technologies (Phoneix, AZ, USA). Both cation-exchange sorbents (150mg/6 mL) – the weak (Oasis WCX) and the strong (MCX) – for the SPE process were obtained from Waters Corp. (Milford, MA, United States).

2.2. Instrumentation

The instrumentation used in this study was an Agilent model 7100 series CE coupled with an Agilent 6460 series triple quadrupole mass spectrometer with an electrospray ionization (ESI) interface from Agilent Technologies (Waldbronn, Germany). For data analysis and instrumental control, Agilent MassHunter Workstation Software (version B.09.00) was used. pH measurements were performed using a GLP 21 pH-meter from Crison (Barcelona, Spain).

2.3. CE-MS/MS conditions

The BGE consisted of 1 M HCOOH:1 M ammonium formate (5:0.1, v/v) with 0.2% sulphated γ-CD. The sheath liquid used in the analyses was a mixture of 10 mM ammonium acetate:MeOH (50:50). The capillary was a bared fused-silica of 50 µm with a total length of 80 cm, which was thermostatted at 25 °C. The conditioning of new capillaries (outside the MS system) consisted of flushing (930 mbar) 1 M NaOH for 40 min followed by 10 min of Milli-Q water. At the beginning of each day, the capillary was conditioned with 0.1 M NH_4OH, followed by Milli-Q water and finally BGE, each for 10 minutes. Between each analysis it was conditioned with 0.1 M NH_4OH, followed by Milli-Q water and finally BGE, each for 5 minutes. The sample was hydrodynamically injected at 50 mbar for 10 s and the separation was performed applying a voltage of 20 kV.

The optimized ion source parameters were as follows: gas temperature, 200 °C; gas flow rate, 2 L/min; nebuliser pressure, 7 psi; and capillary voltage, 4000 V in positive mode. Multiple reaction monitoring (MRM) was the acquisition mode chosen and the two most intensive transitions between the parent and product ions were selected (Table 1). Two different windows were set: the first without acquisition, while the

second acquired the 4 ATS. The fragmentor was set at 100 V for both windows, while the collision energies were between 4 and 15 eV.

2.4. Urine collection and preparation

For the urine analysis, pooled mixed urine samples were obtained from non-addicted laboratory staff, both women and men of different ages. The samples were collected in polypropylene tubes and kept in the freezer at -20 °C. For the urine pre-treatment, an SPE was performed using 150 mg Oasis WCX cartridges. First, they were activated with 5 mL of MeOH and conditioned with 5 mL of phosphate buffer at pH 7 (0.10 M of NaH_2PO_4 and 0.06 M of Na_2HPO_4). Then 25 mL of a mixture of urine diluted with phosphate buffer at the same pH (50:50, v/v) was loaded and washed with 4 mL of MeOH. Finally, the analytes were eluted with 4 mL of 5% HCOOH in MeOH. The extracts were evaporated to dryness under a gentle stream of nitrogen and reconstituted with 0.5 mL of water. They were then filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter prior to their analysis in the CE-MS/MS system.

2.5. Validation

The method validation was performed following European guide-lines for workplace drug testing in urine [37]. It was based on linearity, sensitivity, instrumental detection and quantification limits (IDLs and IQLs), method detection and quantification limits (MDLs and MQLs), apparent recoveries (R_{app}), matrix effect (ME), precision as repeatability (intra-day) and reproducibility (inter-day), selectivity, stability and accuracy. In all cases the retention time had a tolerance of ±2.5% and an ion ratio of ±20% [38].

The instrumental linearity was investigated using different calibrator concentrations of a mixture of ATS neat standards of between 60 and 3500 ng/mL by evaluating the determination coefficient (r^2). IDLs were defined as the lowest detectable point with a signal-to-noise (S/N) ratio ≥3 and IQLs as the lowest concentration in the calibration curve with S/N >10.

The method linearity was studied using a matrix-matched calibration curve of between 2 and 150 ng/mL of a mixture of ATS in urine. The linearity, MDLs and MQLs criteria were the same as for the instrumental parameters, but in this case for extracted samples. The lower values of MDLs and MQLs can be explained because of the precon-centration factor achieved in the SPE procedure. IDLs and IQLs were studied by injecting the standards directly into the CE-MS/MS system, while in the case of MDLs and MQLs, ATS under study were spiked in urine and then, the SPE was carried out. The R_{app}, ME, repeatability and reproducibility were

studied at three different levels of concentration – 10 ng/mL (low), 50 ng/mL (medium) and 100 ng/mL (high) – by using five replicates (n=5). Repeatability and reproducibility were evaluated in terms of relative standard deviation (%RSD), the former being obtained by analysing five replicates on the same day and the latter by analysing one replicate on five different days.

The selectivity and specificity were studied by evaluating the possible endogenous and exogenous interferences that may be present in urine samples from different specimens. The stability was studied by reanalysing the three calibrator levels (10, 50 and 100 ng/mL) in urine every 10 hours for a total of 40 hours at room temperature. The method's accuracy was evaluated in terms of error by analysing three blind samples spiked at different concentrations before all the experimental procedures. After analysing the samples, the concentration of ATS was calculated with the matrix-matched calibration curve and compared to the spiked concentration.

3. Results and discussion

3.1. Separation and detection optimization

The MS/MS parameters were individually evaluated with standard solutions of each ATS at 1 mg/L. Because ATS are basic compounds, the positive mode was used to choose the precursor ion of each analyte. The MRM mode was applied to collect the monitoring and quantitative ions. The fragmentor voltage was investigated between 50 and 200 V and the collision energies between 0 and 30 kV. The two most abundant transitions of each ATS were chosen to confirm their identification. In Table 1, the different precursor and product ions of the analytes are shown at a fragmentor voltage of 100V for all the compounds. The source conditions were optimized for the CE-MS/MS system using the source optimization software, i.e. capillary voltage (4000 V), gas temperature (200 °C), gas flow rate (2 L/min) and nebulizer (7 psi). Finally, two different windows of detection were created, the first without acquisition and the second acquiring the four ATS under study.

Afterwards, the sheath liquid composition and the BGE for the ATS CE separation were evaluated. To this end a mixture of AMP, MAMP and MDMA at 500 ng/mL and an 80 cm bared fused silica capillary of 50 µm were used. These initial experiments were performed without the addition of the chiral selector to avoid possible contamination of the MS detector.

The sheath liquid was studied first, since its composition and flow rate can have a strong influence on the efficiency of the ionization process and detection sensitivity. It can also affect the CE

Table 1. Migration times, precursor and product ions, collision energies and fragmentor voltages of the ATS under study.

Compound	m_t (min)	Precursor ion	Product ion 1	Col. E 1 (eV)	Product ion 2	Col. E 2 (eV)	Product ion 3	Col. E 3 (eV)
R-AMP	15.9	136.1	119	4	91	10	-	-
S-AMP	17.2							
R-MAMP	16.3	150.1	119	8	91	10	-	-
S-MAMP	16.8							
R/S-MDMA	19.1	194.1	163	10	133	15	105	15
R/S-MDMA	20.2							
R/S-MDA	18.7	180.1	163	6	135	10	133	10
R/S-MDA	20.6							

separation process and analyte migration times due to the possible formation of ionic boundaries [39]. Initial experiments to optimize this parameter focused on evaluating its composition. For this, 10 mM ammonium acetate as BGE at 20 kV of separation voltage was used. Under these conditions, two different sheath liquids were evaluated: IPA:H_2O:HCOOH (60:39:1, v/v/v) and 10 mM ammonium acetate:MeOH (50:50), both at a flow rate of 0.4 mL/min. This selection was based on the literature dealing with the determination of ATS by CE-MS [21,23,40]. From the results obtained it was observed that better efficiency and higher peak intensities were obtained with the mixture of 10 mM ammonium acetate:MeOH (50:50), so further experiments were performed with this composition. Afterwards, the optimal flow rate was evaluated by comparing 0.3, 0.4 and 0.5 mL/min and the best peak efficiency was obtained with 0.4 mL/min, so this was the value chosen.

Two different BGEs – 10 mM ammonium acetate and 1 M HCOOH – were evaluated in terms of resolution. This was performed considering their compatibility with the MS system and taking into account that they are both widely employed BGEs in the determination of ATS by CE-MS [21,35,40,41]. Table 2 shows the resolution between the different ATS included in this study using the two aforementioned BGEs. It was observed that the use of formic acid provided better peak resolution than ammonium acetate. Based on these results, a combination of 1 M HCOOH:1M ammonium acetate (5:0.1, v/v) was then tried, and with this BGE the resolution was the same as for 1 M HCOOH. Another experiment involved changing ammonium acetate for ammonium formate in combination with formic acid. This mixture provided lower resolution values than the previous one. Finally, a concentration of 0.5 M formic acid was tried in combination with

ammonium acetate and ammonium formate. With these two BGEs the worst resolution values were obtained and, moreover, the migration times were higher than with the combination of 1 M HCOOH:1M ammonium acetate (5:0.1, v/v). Finally, comparing all the obtained results, we were able to conclude that the best resolution values were obtained with 1M HCOOH and the combination of 1 M HCOOH:1M ammonium acetate (5:0.1, v/v). However, when comparing the peak efficiency values for both, we observed that they were slightly better for the mixture. Therefore, the combination of 1 M HCOOH:1 M ammonium formate was the BGE chosen.

The addition of a chiral selector to the BGE was then evaluated. As mentioned earlier, when using CE-MS/MS it is important to take into account the chiral selector characteristics and use them at the minimum concentration to reduce or avoid their entering the MS system [34]. Although for CE there is a wide variety of chiral selectors, with the most commonly used being native CDs, when the detection system is an MS the choice of these selectors is restricted because, depending on their characteristics, they may cause signal suppression and contamination of the MS detector. Moreover, as mentioned in the Introduction, certain strategies exist to minimize these problems. In the present study CMT was chosen. When using this strategy, it is important to select chiral selectors capable of migrating in the opposite direction to the detector, thereby avoiding their entering the MS since this could produce loss of sensitivity. Moreover, as a complementary tool, during the sample injection the MS ion source can be turned to waste mode to prevent the chiral selectors from entering the ion source [34]. Therefore, to comply with CMT requirements a set of chiral selectors was chosen that had previously been used in various studies in which different ATS were enantioseparated. Specifically, the substances selected for investigation were α-CD sulphated sodium salt, β-CD sulphated sodium salt, γ-CD sulphated sodium salt, γ-CD phosphate sodium salt, β-CD sulphobutylated sodium salt and vancomycin [39,42,43]. Different

Table 2. Resolution of ATS in the BGE optimisation: 10 mM ammonium acetate; 1 M HCOOH; 1 M HCOOH:1 M ammonium acetate (5:0.1, v/v); 1 M HCOOH:1 M ammonium formate (5:0.1, v/v); 0.5 M HCOOH:1 M ammonium acetate (5:0.1, v/v); 0.5 M HCOOH:1 M ammonium formate (5:0.1, v/v).

	10 mM ammonium acetate	1 M HCOOH	1 M HCOOH:1 M ammonium acetate (5:0.1, v/v)	1 M HCOOH:1 M ammonium formate (5:0.1, v/v)	0.5 M HCOOH:1 M ammonium acetate (5:0.1, v/v)	0.5 M HCOOH:1 M ammonium formate (5:0.1, v/v)
AMP - MAMP	0.5	1.7	1.7	1.0	1.4	1.3
MAMP - MDMA	1.0	4.0	4.0	2.3	3.1	3.7
AMP - MDMA	1.5	5.7	5.7	3.3	4.6	4.6

percentages of these chiral selectors ranging between 0.1 and 1% were evaluated. The concentration levels were low to minimize the possible suppression of the ionization of the analytes, which could lead to a severe loss of sensitivity. When using the 0.1% level of chiral selector in the BGE, it was only possible to achieve the enantioseparation of the ATS under study with sulfated γ-CD. However, MAMP was not separated at baseline with this chiral selector at this concentration level. For 0.2%, only with sulfated γ-CD and sulfobutylated β-CD was it possible to achieve the enantioseparation of the analytes, although regarding the latter, the separation between enantiomers was not at baseline. For the higher percentages of all the chiral selectors tested, the peaks were not enantiomerically separated and indeed were distorted. Based on the obtained results, therefore, a combination of 0.1% of sulfated γ-CD with 0.1% of sulfobutylated β-CD was also investigated, and under these conditions it was possible to achieve complete separation between the two enantiomers for each of the ATS under study. The resolution values obtained for the ATS enantiomers with these chiral selectors that provided partial or total enantioseparation are shown in Table 3. Figure 1 shows the electropherograms of the enantio-separation of the ATS using 0.1% sulfated γ-CD (A), 0.1% sulfated γ-CD + 0.1% sulfobutylated β-CD (B), 0.2% sulfated γ-CD (C) and 0.2% sulfobutylated β-CD (D).

It can be seen that the best resolution results were obtained with 0.2% of sulfated γ-CD. For this CD the values were the higher for AMP and MDMA, whereas for MAMP the resolution obtained was the same as or similar to that obtained with the combination of 0.1% of sulfated γ-CD with 0.1% of sulfobutylated β-CD or 0.2% sulfobutylated β-CD. Therefore, 0.2% of sulfated γ-CD was chosen as the optimum chiral selector in the present study.

Figure 2 shows an electropherogram using the optimal conditions to determine the ATS, including in this case MDA. As can be seen, the four ATS were totally enantioseparated using the CMT approach in combination with sulfated γ-CD. Moreover, with the use of R and S standards for AMP and MAMP, the R-enantiomer migrated earlier than the S. However, due to the lack of R/S standards for MDMA and MDA, the identification of each enantiomer could not be performed for these two compounds.

3.2. SPE optimization

For the urine pre-treatment, SPE was the strategy using cation-exchange sorbents selected due to the amino group of ATS, which are charged at a

Table 3. Resolution between enantiomers of AMP, MAMP and MDMA with different chiral selectors at 500 ng/mL.

	AMP	MAMP	MDMA
0.1% sulphated γ-CD	2.7	0.7	1.7
0.1% sulphated-γ-CD + 0.1% sulphobutylated-β-CD	2.7	1.3	1.8
0.2% sulphated γ-CD	3.3	1.3	2.4
0.2% sulphobutylated β-CD	1.0	1.2	1.5

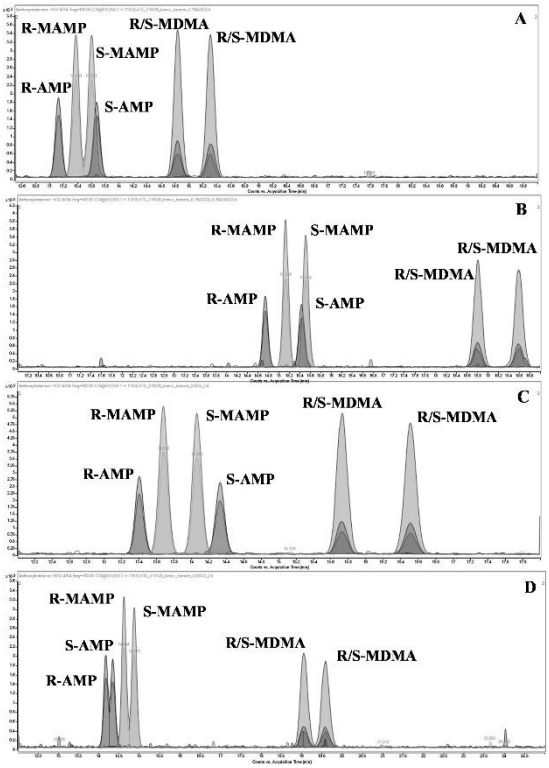

Figure 1. Comparison of different chiral selectors: 0.1% of sulfated γ-CD (A), a combination of 0.1% of sulfated γ-CD with 0.1% of sulfobutylated β-CD (B), 0.2% of sulfated γ-CD (C), and 0.2% of sulfobutylated β-CD (D) with a mixture of AMP, MAMP, and MDMA at 500 ng/mL with a BGE of 1 M HCOOH:1 M ammonium formate and with a sheath liquid of 10 mM ammonium acetate:MeOH (50:50) at 0.4 mL/min.

lower pH than their pK_a. To this end Oasis WCX (weak) and Oasis MCX (strong) sorbents were compared for the SPE process. The methodologies for both SPE sorbents were adopted from a previously published paper by our group regarding the determination of cathinones by LC-HRMS [44]. In the present study, the SPE procedure for both sorbents was first optimized by using urine samples spiked at 200 ng/mL of each analyte to enable us to select the sorbent that provided better results in terms of recovery.

In the case of Oasis WCX, this was first activated with 5 mL of MeOH and conditioned with 5 mL of phosphate buffer at pH 7 (0.10 M of NaH_2PO_4 and 0.06 M of Na_2HPO_4). A mixture of 5 mL of urine:phosphate buffer (pH 7) (50:50, v/v) was then loaded, washed with 2 mL of MeOH and finally eluted with 2 mL of 5% HCOOH in MeOH. Following this protocol, recoveries of around 95% for all the analytes were obtained. As for the Oasis MCX cartridge, the activation and conditioning consisted of 5 mL of MeOH followed by 5 mL of phosphate buffer at pH 6 (0.15 M NaH_2PO_4 and 0.05 M Na_2HPO_4) and the loading of 5 mL of a mixture of urine:phosphate buffer (pH 6) (50:50, v/v). It was then washed with 2 mL of MeOH and eluted with 2 mL of 5% NH_4OH in MeOH. With this protocol, recoveries around 90% for all analytes were achieved.

Although similar recoveries were obtained with both sorbents, Oasis WCX was selected for further study due to its slightly higher values (around 5% and 10% higher). As our aim was to determine low concentration levels of ATS in urine samples and bearing in mind that CE used techniques and has a

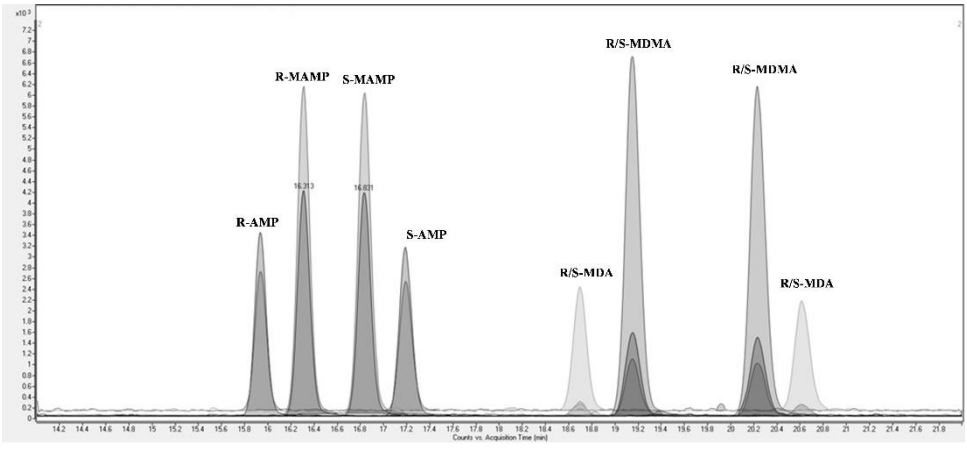

Figure 2. Electropherogram of a mixture of AMP, MAMP, MDMA, and MDA at 1000 ng/mL analyzed by CE-MS/MS with a BGE of 1 M HCOOH:1 M ammonium formate with 0.2% of sulfated γ-CD and a sheath liquid of 10mM ammonium acetate:MeOH (50:50) at 0.4 mL/min.

lower sensitivity than LC, the SPE process was reoptimized to gain greater preconcentration of the analytes, thereby enabling lower limits of concentration to be achieved. To this end the sample volume was increased and 25 mL of urine:phosphate buffer (pH 7) (50:50, v/v) evaluated. As the volume of urine was increased, the washing and elution steps were also reoptimized by increasing their volumes. A study was made of 4 mL of MeOH and 4 mL of 5% HCOOH in MeOH, respectively, for washing and eluting. Under these conditions no losses in the washing step were observed and 4 mL of MeOH was chosen as the optimal washing volume. Increasing the elution solvent from 2 mL to 4 mL led to an increase of 15% in the $\%R_{app}$, so 4 mL was chosen as the optimal volume. The final conditions for Oasis WCX were therefore the activation and conditioning with 5 mL of MeOH and 5 mL of phosphate buffer at pH 7, then loading 25 mL of a mixture of urine:phosphate buffer (pH 7), washing with 4 mL of MeOH, and finally eluting with 4 mL of 5% HCOOH in MeOH. Under these conditions a $\%R_{app}$ of between 70% and 82% was achieved when a urine sample containing the ATS at a concentration of 10 ng/mL was analysed.

3.3. Method validation

The method using Oasis WCX was validated with urine samples by evaluating linear range, MQLs, MDLs, repeatability (intra-day), reproducibility (inter-day), stability and accuracy.

The proposed method presented IDLs from 20 to 40 ng/mL and IQLs between 60 and 125 ng/mL for the ATS under study. The $\%R_{app}$, %ME, repeatability and reproducibility were studied at low (10 ng/mL), medium (50 ng/mL) and high (100 ng/mL) levels of ATS concentration in urine (Table 4). Different blanks of urine samples from different laboratory staff members demonstrated good selectivity of the method because no endogenous or exogenous interferences were observed at the same migration times of the ATS. The exogenous interferences studied were cocaine and a group of synthetic cathinones.

Matrix-matched calibration curves were chosen as the calibration model. The linear range was from the MQL to 150 ng/mL for all the compounds, with r^2 higher than 0.990 for all ATS under study. The MDLs in urine were between 0.6 and 1.5 ng/mL, while the MQLs ranged from 2.0 to 8.0 ng/mL. It is important to note that these values are suitable for reaching the levels at which ATS are usually found in urine (ng/mL). Values from 70% to 82%, from 80% to 90% and from 82% to 95% were achieved for $\%R_{app}$ at low (10 ng/mL), medium (50 ng/mL) and high (100 ng/mL) levels of concentration in urine respectively. For %ME, the values

obtained at the same three concentration levels were between -26% and -15%, -17% and -10% and -14% and -6% respectively. In addition, values below 20% of %RSD were obtained for repeatability (intra-day) and reproducibility (inter-day) at the levels of concentration stated above. The analytes were shown to be stable at room temperature for 40 h with no evidence of degradation and with RSDs lower than 7% for each calibration level for the different periods of time evaluated (every 10 h).

The method was finally tested to prove its accuracy by analysing three blind urine samples spiked with the ATS under study at 14 ng/mL, 38 ng/mL and 75 ng/mL. Accuracy was calculated as the percentage of error between the concentration found when analysing these samples per triplicate and the spiked concentration in the same sample. Errors were between 12% and 16% for the first sample, 7% and 14% for the second and 4% and 12% for the third.

The developed method is comparable to other previously published studies in which different strategies based on SFC, LC-MS/MS, LC-QTrap or GC-MS were used [11,13,14,27,28]. It is important to highlight that in the present study, lower MDLs and MQLs are achieved compared to some of these earlier studies. For example, Hegsatd et.al. [11] developed a method to quantify R/S-AMP in urine by SFC-MS/MS with semi-automatic sample extraction. They achieved MDLs of 10 ng/mL and MQLs of 25 ng/mL. Other authors who used LC-MS/MS as the analytical technique to determine ATS in urine samples also obtained higher MDLs and MQLs than those obtained in the present study [13,14,28]. Although these limits are higher than those obtained in the present study, they used considerably lower loading volumes, less than 1 mL, even though urine is not a problematic matrix considering its availability in huge quantities, so using more volume is not an issue. However, there are other

Table 4. Validation parameters for the determination of ATS in urine by CE-MS/MS

Analyte	MDLs[a]	MQLs[a]	$\%R_{app}$ (n=5)			%ME (n=5)			Repeatability %RSD (n=5)			Reproducibility %RSD (n=5)		
			Low[b]	Medium[c]	High[d]	Low[b]	Medium[c]	High[d]	Low[b]	Medium[c]	High[d]	Low[b]	Medium[c]	High[d]
R-AMP	1.0	5.0	78	85	88	-18	-12	-11	8	1	10	8	3	2
S-AMP	1.0	5.0	80	86	93	-17	-12	-8	10	2	11	2	3	4
R-MAMP	1.0	8.0	82	90	95	-15	-10	-6	2	2	6	5	5	3
S-MAMP	1.0	8.0	80	84	94	-17	-13	-7	6	5	8	9	4	3
R/S-MDMA	0.6	2.0	76	86	94	-21	-10	-8	9	5	7	13	8	8
R/S-MDMA	0.6	2.0	79	85	95	-18	-13	-7	5	6	12	10	8	8
R/S-MDA	1.5	8.0	74	83	86	-22	-14	-11	18	4	11	11	2	4
R/S-MDA	1.5	8.0	70	80	82	-26	-17	-14	15	6	16	14	10	6

[a] ng/mL [b] 10 ng/mL [c] 50 ng/mL [d] 100 ng/mL

examples in which the reported MDLs were lower, such as the method developed by Fujii et al. [27], in which AMP, MAMP, MDA, MDMA and other ATS were enantiomerically separated in less than 5 min. Nevertheless, one of the strengths of our method is that these methodologies use more expensive equipment, whereas in the present case the enantioseparation is easily achieved with the simple addition of a chiral selector to the BGE. Shorter analysis times are also involved in our strategy, and lower organic solvent volumes are used compared to the strategies based on SFC, GC or LC mentioned above.

4. Conclusions

A method for the enantio-determination of a group of ATS in urine by CE-MS/MS was successfully developed and validated. In the present case, using the CMT with a BGE of 1 M HCOOH:1 M ammonium formate with 0.2% of sulphated γ-CD, 4 ATS including parent compounds and some of their metabolites were enantioseparated. This proves the strength of the CE technique in combination with MS, achieving low levels of concentrations with easy enantiomeric separation. In addition, two different cationic-exchange SPE sorbents were compared in the urine pre-treatment, with Oasis WCX achieving better results compared to Oasis MCX in terms of Rapp. Finally, the method showed that it can determine both enantiomers of the four compounds studied both at low levels of concentration and at the usual levels in which they can be present in urine from drug users. It is therefore demonstrated that this method is a useful tool in toxicological and forensic analyses.

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Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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3.2.2. Discussion of results

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In the present section, the most important results obtained from the enantiodetermination of four ATS in urine by SPE followed by CE-MS/MS are discussed.

The selection of the compounds studied was based on the most common ATS and their metabolites. AMP, MAMP and MDMA are the most consumed ATS and some of the most popular over the world. Regarding their metabolites, MDA is the metabolite of MDMA and MAMP is metabolised in AMP. Therefore, AMP can be found due to its consumption or to the metabolization of MAMP [1]. In the SPE procedure, as these compounds are positively charged at a pH under their pK_a, Oasis WCX and Oasis MCX were evaluated and compared [2]. Both of them achieved similar $\%R_{app}$ but the obtained with Oasis WCX were slightly lower (between 5 and 10%). In particular the obtained $\%R_{app}$ were between 70 and 95% for all the compounds.

The enantiomeric separation using CE-MS is still challenging because not all the BGEs and chiral selectors can be used, as they can contaminate the MS source. For that reason, different volatile BGEs were studied. In particular, ammonium acetate, HCOOH, ammonium formate and different combinations of ammonium formate/acetate with HCOOH were also investigated in terms of resolution and peak efficiency. It could be observed that the best resolution between ATS was obtained with 1 M HCOOH and a combination of 1 M HCOOH with 1 M ammonium acetate. However, the combination of 1 M HCOOH with 1 M ammonium formate achieved the best peak efficiency of the analytes. Therefore, as the resolution provided by this last BGE was enough to separate all ATS and because the peak efficiency was higher than the other, the mixture of 1 M HCOOH with 1 M ammonium formate was selected as the optimal BGE. Then, different chiral selectors were evaluated using the CMT approach to avoid their entrance into the MS. In particular, α-CD sulfated sodium salt, β-CD sulfated sodium salt, γ-CD sulfated sodium salt, γ-CD phosphate sodium salt, β-CD sulfobutylated sodium salt, and vancomycin, which are some of the most used by the authors [3–5]. Different percentages of these chiral selectors were studied (between 0.1 and 1%). Results showed that the enantioseparation of the ATS under study could be obtained with 0.1% and 0.2% of sulphated γ-CD, 0.2% of sulfobutylated β-CD and with a combination of 0.1% of sulphated γ-CD and 0.1% of sulfobutylated β-CD. However, 0.1% of sulphated γ-CD and the combination of 0.1% of sulphated γ-CD

and 0.1% of sulfobutylated β-CD could not separate all the enantiomers at baseline. The resolution between the enantiomers of AMP, MAMP and MDMA by using these different chiral selectors was also studied, showing that 0.2% of sulphated γ-CD achieved the best resolution for all the enantiomers. For that reason, 0.2% of sulphated γ-CD was selected as the optimal, being able to separate the 8 enantiomers in 5 minutes in a total analysis time of 22 minutes. Moreover, low MDLs and MQLs were also achieved (between 0.6 and 8.0 ng mL^{-1}), which are suitable to detect these compounds in urine specimens from drug users [6,7]. One of the main reasons of the high sensitivity achieved with the CE instrumentation is due to its coupling with a QqQ.

Therefore, this methodology shows the strength of using CE for the enantiomeric separation, as by the simply addition of small amounts of a chiral selector, the enantioseparation is easily achieved and the compatibility problems of this kind of substances with the MS are solved by using charged CDs with approaches such as CMT.

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3.3. Determination of classic and synthetic drugs in urine from drug abusers

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As it has been described in the Introduction, there are different families of DOAs such as cannabis, COC, ATS, opioids, NPS and other DOAs. Even that every year new NPS are appearing into the illegal market, the classic drugs as ATS, COC, BZD and some opioids are still the most consumed [1–3]. Therefore, it is important to provide methodologies able to determine not only these new substances but also the classic ones. Moreover, depending on the matrix, it is also important to include the parent drugs and their metabolites, because the presence of the metabolite can indicate a drug consumption in the absence of the parent drug [4].

The determination of DOAs in toxicological and forensic studies is very important and one of the most employed matrices is urine. The main purposes in this field are the obtention of multiresidue and sensitive methodologies with special attention to easy, fast and green strategies [5–7]. As in the previous sections the methods were focused on specific DOAs families as synthetic cathinones and ATS, the present section aims to develop a multitarget method determining these two groups together with other families of DOAs and some of their metabolites in urine.

In particular, the first study was focused on the comparison of four different mixed-mode cationic exchange sorbents for the SPE pre-treatment of urine to achieve a highly sensitive methodology using LC-QqQ. The four SPE sorbents evaluated and compared were: Oasis MCX, Oasis WCX, ExtraBond ECX and ExtraBond SCX. The two first were previously evaluated for synthetic cathinones and ATS, while the others are similar sorbents with the same characteristics but from another commercial brand. All of them were selected because they are mixed-mode sorbents that usually achieve high extraction efficiencies with exhaustive clean-up steps: Considering that most DOAs under study are basic compounds, by using a pH lower than their pK_a, high extraction efficiency can be obtained [8,9].

In order to achieve an easy, fast and a green strategy, the second study was based on the miniaturisation of the SPE performed in the previous study using a pipette-tip SPE (PT-SPE) for the determination of the same DOAs and metabolites in urine. This miniaturised technique is based on the extraction of the analytes in a tip that contains the SPE sorbent. This allows the easy extraction of the analytes

by only using a pipette, which can be filled by the user with commercial or home-made sorbents, or also there are some commercially prepared pipette tips. This strategy has been previously used to determine different DOAs achieving good extraction efficiencies [10]. Even though, most of the reported literature in this field is focused on a particular family of DOAs or in few substances. For that reason, the present study aims to develop a home-made PT-SPE device to determine a large number of DOAs from different families and their metabolites in urine. Special attention has been paid to each extraction step to achieve the highest extraction efficiency. In particular, parameters such as loading, washing and elution volume or aspirating/dispensing cycles have been evaluated to achieve the highest recoveries of all the compounds [10,11].

Moreover, to demonstrate the applicability of the developed strategies, both studies have been applied for the determination of DOAs in urine from women starting a detoxification program from the *Centre Català de la Salut (CECAS)* in Tarragona, Spain. The first study has been published in the scientific journal Journal of Analytical Toxicology (in press) and the second is under revision in Journal of Chromatography A and they are presented in section 3.3.1. and 3.3.2., respectively, in scientific article format.

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3.3.1. Development of a liquid chromatography-tandem mass spectrometry method for the simultaneous determination of 40 drugs of abuse in human urine: application to real cases

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Development of a Liquid Chromatography-Tandem Mass Spectrometry Method for the Simultaneous Determination of 40 Drugs of Abuse in Human Urine: Application to Real Cases

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Abstract

Drugs of abuse are constantly evolving, while new synthetised substances are constantly emerging to avoid regulations. However, traditional drugs such as cocaine and amphetamine are still two of the most consumed drugs in the world. It is important, therefore, to provide suitable multiresidue methods for determining a wide range of drugs for use in toxicological and forensic analyses. The aim of this study is to develop a method for determining several families of drugs of abuse, including classic drugs, new psychoactive substances and some of their metabolites, in urine by liquid chromatography-tandem mass spectrometry. Urine is one of the most common biological matrices used in drug analysis because of its easy collection and wide window of detection. In this study, we used solid-phase extraction to remove interferences and extract analytes from urine. Four different mixed-mode cation exchange commercial sorbents were evaluated. The best results, in terms of apparent recoveries, were achieved with one of the strong cationic sorbents, i.e. ExtraBond SCX. The method achieved detection limits from 0.003 to 0.500 ng/mL and quantification limits from 0.050 to 1.500 ng/mL, which are suitable for determining these compounds at the usual levels found in the urine of drug users. Applicability of this method was demonstrated by analysing real urine specimens from women following a detoxification program. Our results showed that the drug most consumed was cocaine, since it was detected in most urine specimens together with its main metabolite, benzoylecgonine. The polyconsumption of drugs from different families was also observed in some urine samples analysed.

Keywords: *Cation-exchange sorbents · LC-MS/MS · Human urine · Toxicological Analysis · Drugs of abuse*

Introduction

The last few years have seen the wide availability of numerous substances on the drugs market. As well as traditional compounds, there has been a high demand for more potent drugs, which is where synthetic drugs play an important role. Moreover, the prohibition of certain substances has led to the consolidation of illegal markets. Hundreds of new psychoactive substances (NPS), especially stimulant substances, have been synthetized (1). In view of these new substances, as well as the classic drugs already on the global market, drugs of abuse (DOAs), are now widely available. These can be classified in different ways. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported the following classification in its annual report: cannabis, cocaine (COC), amphetamine-type substances (ATS), NPS, heroin (HER) and other opioids, and other drugs such as ketamine (KET) and lysergic acid diethylamide (LSD) (2). According to the EMCDDA, the amounts of COC seized in Europe are now the highest ever seen and this drug has become the second most consumed illicit drug in Europe after cannabis. Another well-known group is ATS, which contains substances such as amphetamine (AMP), methampheta-mine (MAMP) and 3,4-methylenedioxy-methammphetamine (MDMA or ecstasy) (3). In addition to ATS, COC and cannabis, other substances also consumed in Europe are LSD, KET and NPS. The latter is a heterogeneous group of synthetic drugs in which the most detected substances are synthetic cannabinoids, synthetic cathinones and synthetic opioids (4, 5). Control of DOAs is a matter of concern and Spain is an important country for its interception because it is one of the main gateways for the entry of COC and cannabis into Europe (3–5). Several studies in Spanish wastewaters, for example, have revealed an increase in ATS and COC consumption and high levels of COC metabolites in wastewater samples (6–8).

These substances are controlled by medical and government authorities, who are interested in developing reliable multiresidue methods that can simultaneously determine a large number of DOAs, including classic drugs and new synthetic substances. In forensic and toxicological analyses, their determination is important in biological matrices such as urine, blood, oral fluid or hair (4, 9, 10). Urine has several advantages over other biological matrices because it provides a wide window of detection (from minutes to days or weeks), in general, no specialized personnel is required for the sampling, and the amount of sample produced is very high. It is also important sensitive methods able to determine both the parent drugs and their metabolites, which can be found between low and high levels of ng/mL.

Urine also has several drawbacks, however, including easy adulteration when the collection is not observable and invasive sampling when it is (9–15).

The complexity of urine makes it important to conduct sample pre-treatment before analysis because possible interferents with the analytes of interest can be problematic (9, 10, 15). One of the most common sample pre-treatments in the literature is solid phase extraction (SPE). This has been used by numerous authors with methods for determining various types of DOAs with different SPE sorbents (16–20). Since most drugs are basic compounds with pK_a values above 7, at a lower pH they are positively charged and mixed-mode cationic-exchange sorbents can therefore be perfect candidates (9, 19). A wide range of commercial cation-exchange SPE sorbents exists. It is important to choose the one that provides the highest recovery efficiencies for most of the compounds studied. In this paper, we compare four different commercial mixed-mode cation-exchange sorbents for sample pre-treatment.

To analyse the extracted urine analytes, various techniques can be used, including liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (9). The most common of these is LC because of the polar characteristics of the DOAs under study. In general, LC has been combined with mass spectrometry (MS) and, more precisely, with tandem MS (MS/MS) using a triple quadrupole (QqQ) or QTrap or with LC-HRMS by using Orbitrap to obtain sensitive and selective methods (14, 16–19, 21–28). Concheiro et al. (25), for example, developed a method for determining 40 novel psychoactive stimulants in urine by LC-QOrbitrap using SOLA SCX cartridges for the SPE urine pre-treatment, and achieved extraction efficiencies between 90% and 100% for most compounds. The authors obtained limits of detection (LODs) between 1.0 and 5.0 ng/mL and limits of quantification (LOQs) between 2.5 and 5.0 ng/mL. For their part, Rosano et al. (14), Ambach et al. (24) and Di Trana et al. (26) used LC-MS/MS to determine DOAs in urine using sample pre-treatment procedures such as hydrolysis, liquid-liquid extraction (LLE) and dilute-and-shoot, respectively. In particular, when Di Trana et al. (26) analysed urine specimens from drug users, they determined MOR (180 ng/mL), several synthetic ATS (e.g. 6-APB from 3.5 to >10000 ng/mL) and several synthetic cathinones (e.g. mephedrone between 12.4 and 3597 ng/mL), among other substances. These results and those of similar studies show the wide concentration ranges at which these substances can be found in urine and the need for sensitive methods to determine them. Despite the good results generally obtained with the

above methods, most strategies focus mainly on a low number of drugs generally belonging to the same family. It is important, therefore, to provide methods that can simultaneously determine different classes of drugs, including classic drugs and the new synthetic drugs, because poly-consumption is very common among drug users. The main aim of this study is to contribute to the literature with a fast and sensitive method for the multiclass determination of different types of DOAs and some of their metabolites in urine by SPE followed by LC-QqQ.

This study focuses on some of the most consumed classic illicit drugs and some NPS (e.g., synthetic cathinones). To extract these drugs from urine and perform clean-up, we tested and compared for the first time several types of mixed-mode cation exchange sorbents (both weak and strong), including Oasis WCX, Oasis MCX, Extrabond ECX and ExtraBond SCX. Finally, to prove that this is a useful method in toxicological cases we applied it to urine samples from women following a detoxification program.

Experimental

Standards and materials

The following drug standards were purchased from LGC Standards (Luckenwalde, Germany) or Sigma Aldrich (St. Louis, MO, USA): 4-fluoromethcathinone (flephedrone), N-ethylcathinone (ethcathinone), buphedrone, 2-methylmethcathinone (2-MMC), butylone, mephedrone, 4-methylethcathinone (4-MEC), beta-ethylmethcathinone (pentedrone), 3,4-dimethylmethylcathinone (3,4-DMMC), alpha-pyrrolidinovalerophenone (alpha-PVP), methylenedioxypyrovalerone (MDPV), 3,4-methylenedioxymethcathinone (methylone), 4-methxymethcathinone (methedrone), 3,4-methylenedioxy-N-ethylcathinone (ethylone), dimethylcathinone (DMC), pyrovalerone, AMP, MAMP, MDMA, 3,4-methylenedioxyamphetamine (MDA), COC, benzoylecgonine (BZE), morphine (MOR), 6-acetylmorphine (6-AM), buprenorphine, HER, fentanyl, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), codeine (COD), alprazolam, lorazepam, diazepam, bromazepam, KET, norketamine, LSD, oxo-LSD, 4-methylephedrine and hyoscine.

Depending on the compound, individual stock solutions at 100 mg/L, 1000 mg/L, and 2000 mg/L were prepared in methanol (MeOH) and frozen at -20°C. A mixture solution at 2 mg/L in MeOH was also prepared with all the individual standards and kept in the freezer. From this mixture, diluted working solutions in water (H_2O) were further prepared.

H_2O and acetonitrile (ACN) for LC-MS were obtained from Scharlab (Barcelona, Spain). MeOH was purchased from

J.T. Baker (Deventer, The Netherlands). Formic acid (HCOOH) ≥98%, hydrochloric acid (HCl) ≥37%, ammonium hydroxide (NH_4OH), sodium dihydrogen phosphate (NaH_2PO_4), and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Ultrapure water was obtained from a water purification system (Merck Millipore, Darmstadt, Germany).

Several extraction cartridges for SPE were evaluated. Oasis WCX and MCX (150 mg/6 mL) were purchased from Waters Corp. (Milford, MA, USA). ExtraBond ECX (200 mg/6 mL) and ExtraBond SCX (1000 mg/6mL) were purchased from Scharlab. A MiVAc Duo sample concentrator from Genevac (Ipswich, UK) was used to evaporate the solutions.

LC-MS/MS conditions

For the analysis we used an Agilent model 1200 series LC coupled with an Agilent 6460 series triple quadrupole mass spectrometer with an electrospray ionisation (ESI) interface from Agilent Technologies (Waldbronn, Germany). Agilent MassHunter Workstation Software version B.09.00 was used for instrumental control and data analysis.

The column used for chromatographic separation was a Luna Omega 5 µm Polar C_{18} (150 mm x 4.6 mm, 5 µm) from Phenomenex (Torrance, CA, United States) with a Security Guard from Phenomenex. A mobile phase flow rate of 0.6 mL/min was used with an injection volume of 10 µL. The mobile phase was A: 0.1% HCOOH in H_2O and B: 0.1% HCOOH in ACN in gradient mode. The gradient was as follows: initial 15% B was maintained for 5 minutes before increasing to 35% in 7 minutes and to 80% in 13 minutes. It was then increased to 100% in 1 minute and held for 2 minutes before returning to the initial conditions in 1 minute, where it was maintained for 5 minutes.

The MS/MS parameters were optimised by injecting each compound at 1 mg/L in a mixture of H_2O:MeOH (50:50, v/v). The acquisition was performed in Multiple Reaction Monitoring (MRM) mode in positive polarity. The drugs were divided into ten different windows depending on their retention times (Rt). The optimal MS/MS parameters were as follows: gas temperature, 350ºC; gas flow rate, 13 L/min; nebulizer pressure, 30 psi; and capillary voltage 2500V. The fragmentor was set between 50 and 125 V, and the collision energy (CE) was set between 4 and 42 eV. The two most intensive transitions between the parent ion and the product ions were selected for the MRM mode (**Table I**).

Urine collection and preparation

Urine samples were collected in polypropylene tubes and kept in the

freezer at −20 °C. Before analysis, the samples were thawed and 5 mL of a mixture of urine and ultrapure water adjusted to pH 4 with HCOOH ≥98% at a ratio of 50:50 (v/v) was loaded into a 1.5 g ExtraBond SCX cartridge after activation with 5 mL of MeOH and conditioning with 5 mL of ultrapure water at pH 4. This was then washed with 2 mL of MeOH and the analytes were eluted with 3 mL of 5% NH_4OH in MeOH. After the SPE procedure, 100 µL of 1% HCl ≥ 37% in MeOH was added to the methanolic solution and the extracts

Table I. MRM parameters of the DOAs under study.

	Compound	Rt (min)	Precursor ion	Product ion 1	CE 1 (eV)	Product ion 2	CE 2 (eV)	Product ion 3	CE 3 (eV)	Fragmentor voltage (V)
1	MOR	3.55	286.1	165	40	153	40	157	40	50
2	COD	5.50	300.2	165	40	199	36	153	42	100
3	Methylephedrine	5.91	180.0	162	8	147	10	131	10	100
4	DMC	6.03	178.1	105	18	133	14	72	18	100
5	Methylone	6.36	208.0	160	16	132	20	190	10	100
6	Flephedrone	6.47	182.1	164	15	149	15	123	15	100
7	AMP	6.53	136.1	119	4	91	10	-	-	100
8	Ethcathinone	6.61	178.1	160	12	132	14	105	18	100
9	Hyoscine	6.87	304.0	156	20	138	20	121	20	100
10	6-AM	7.31	328.2	211	36	193	36	165	40	100
11	MDA	7.42	180.1	163	6	135	10	133	10	100
12	Methedrone	7.80	194.1	176	10	161	16	145	16	100
13	MAMP	7.82	150.1	119	8	91	10	-	-	100
14	Ethylone	7.95	222.1	174	18	204	12	146	18	100
15	Buphedrone	8.17	178.1	91	15	131	15	132	15	100
16	MDMA	8.78	194.1	163	10	133	15	105	15	100
17	Oxo-LSD	9.33	356.0	237	20	222	20	265	12	100
18	2-MMC	9.63	178.1	160	8	145	15	119	15	100
19	Butylone	9.82	222.1	174	14	204	10	191	10	100
20	Mephedrone	10.19	178.1	160	8	145	15	119	15	100
21	Norketamine	10.65	224.0	207	8	179	12	125	12	100
22	KET	11.54	238.0	125	20	220	12	207	12	125
23	BZE	11.78	290.1	168	20	105	20	82	20	125
24	4-MEC	11.92	192.1	174	10	146	16	145	18	125
25	Pentedrone	12.52	192.1	174	10	132	16	91	18	125
26	3,4-DMMC	13.58	192.1	174	10	159	15	133	15	125
27	HER	14.03	370.0	211	36	268	36	165	42	125
28	Alpha-PVP	14.23	232.2	91	22	105	25	126	25	125
29	COC	14.75	304.2	182	18	105	22	82	22	125
30	MDPV	14.84	276.2	126	25	135	25	175	20	125
31	LSD	15.53	324.2	231	24	208	26	180	26	125
32	Pyrovalerone	16.51	246.2	105	22	126	26	119	26	75
33	Bromazepam	17.37	316.0	182	30	209	30	288	30	100
34	Fentanyl	17.62	337.2	188	22	105	24	-	-	100
35	Buprenorphine	18.33	468.0	55	48	396	42	414	35	100
36	EDDP	19.03	278.0	234	30	249	20	186	30	100
37	Methadone	19.93	310.2	265	12	105	18	-	-	100
38	Lorazepam	20.42	321.0	275	20	303	10	230	20	100
39	Alprazolam	21.21	308.4	281	24	274	24	204	30	100
40	Diazepam	23.05	285.0	193	30	154	30	222	30	75

were evaporated to dryness with a MiVac sample concentrator. Finally, the extracts were reconstituted with 0.5 mL of mobile phase at initial conditions, filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter, and transferred to a vial for analysis.

Validation

The method was validated by following the European guidelines for workplace drug testing in urine (29). The validated parameters were linearity, sensitivity, instrumental detection and quantification limits (IDLs and IQLs), method detection and quantification limits (MDLs and MQLs), apparent recoveries (R_{app}), matrix effect (ME), precision as repeatability (intra-day) and reproducibility (inter-day), selectivity, stability and accuracy. Tolerances of ±2.5% and ±20% were also considered for retention times and ion ratios, respectively (30).

Instrumental linearity was evaluated by using different working solutions of drug neat standards between 0.010 and 250 ng/mL and considering the determination coefficient (r^2). IDLs were defined as the lowest detectable point with a signal-to-noise (S/N) ratio ≥3. IQLs were defined as the lowest concentration in the calibration curve with S/N ≥10.

Method linearity was studied by using matrix-matched calibration curves with a mixture of drugs in urine at concentrations between 0.010 and 125 ng/mL. The criteria for method linearity, MDLs and MQLs were the same for the extracted samples as they were for the instrumental values. Rapp, ME, repeatability and reproducibility were studied at 2 ng/mL (low), 20 ng/mL (medium) and 65 ng/mL (high) concentration levels using five replicates (n=5). Repeatability (n=5 on the same day) and reproducibility (n=5 for five days) were studied in terms of relative standard deviation (%RSD).

Selectivity and specificity were studied by evaluating possible endogenous and exogenous interferences from urine. Stability was studied by analysing replicates of the three calibrator levels during the sequence (2, 20 and 65 ng/mL) in urine for 50 h at room temperature (n=5). Accuracy was studied in terms of error by analysing three blind samples spiked at various concentrations before conducting the experimental procedure and comparing the concentrations obtained to the real ones.

Analysis of real samples

Urine samples from 22 anonymous women who began a drug-abuse detoxification process were provided by the Centre Català de la Solidaritat (CECAS) in Tarragona, Spain. All samples were collected in polypropylene tubes when the patient was admitted. The

samples were then frozen at -20°C before the sample pre-treatment step described earlier was conducted.

Results and discussion

Separation and detection

Forty compounds, including DOAs and some of their metabolites, were separated with a Luna Omega 5 µm Polar C_{18} column (150 mm x 4.6 mm i.d., particle size 5 µm). The metabolites included in this study were: 4-methylephedrine (metabolite of mephedrone), AMP (MAMP), MDA (MDMA), BZE (COC), norketamine (KET), EDDP (methadone), 2-oxo-LSD (LSD), and 6-AM (HER). The mobile phase comprised A: 0.1% HCOOH in H2O and B: 0.1% HCOOH in ACN. This selection was based on studies previously conducted by our research group in which several synthetic cathinones were determined in various biological matrices (19, 31). The elution gradient was also based on the strategies employed in those studies. However, as the present study included more DOAs, it was modified to obtain the most adequate separation of all the compounds. The mobile phase therefore began at 15% B, which was maintained for 5 minutes before increasing to 35% in 7 minutes. This was increased to 80% in 13 minutes and then to 100% in 1 minute, where it was held for 2 minutes before returning to the initial conditions in 1 minute and being held for 5 minutes. The flow rate for the mobile phase was 0.6 mL/min with and injection volume of 10 µL. Under these chromatographic conditions, most DOAs were separated in less than 25 minutes.

To evaluate the MS/MS parameters, we used standard solutions of each drug at 1 mg/L. The fragmentor voltage was tested between 50 and 200 V and the collision energies (CE) were tested between 0 and 50 kV. The two most abundant transitions of each drug were chosen to confirm their identification in positive mode (**Table I**). The source conditions, i.e. capillary voltage (2500 V), gas temperature (350 °C), gas flow rate (13 L/min) and nebulizer (30 psi), were then evaluated using the Source optimizer software. Once the MRM method was optimised, ten windows of detection were created in accordance with retention times and acquisition.

SPE sorbent selection

In the present study, we tested and compared for the first time four mixed-mode cation-exchange sorbents (both weak and strong) for extracting DOAs and some of their metabolites from urine. In particular, we used one weak (Oasis WCX) and three strong (Oasis MCX, ExtraBond ECX and ExtraBond SCX) sorbents. The main differences were the stationary phase and the amount of sorbent used (150, 150, 200 and 1000 mg, respectively). All these

sorbents are based on polymeric phases – except ExtraBond SCX, which is based on a silica phase. These extraction sorbents were chosen by taking into account the characteristics of the compounds under study since most of these were basic with pK_a values ranging from 7 to 10. However, the pK_a values of benzodiazepines (BZD) are roughly 2, except for lorazepam and bromazepam, which range from 7 to 10. Some of the sorbents used have already been tested for determining synthetic cathinones (19) and ATS in urine (32), with extraction efficiencies above 80% for Oasis WCX and Oasis MCX. The protocols for the two Oasis sorbents were the same as those used in the above study on synthetic cathinones because they are also basic compounds. Specifically, for Oasis MCX the procedure was as follows: 5 mL of MeOH, 5 mL of phosphate buffer at pH 6 (conditioning), 5 mL of urine: phosphate buffer pH 6 (loading), 2 mL MeOH (washing) and 2 mL 5% NH_4OH in MeOH (elution). For Oasis WCX, the procedure involved conditioning with MeOH and phosphate buffer at pH 7 before the sample was loaded at pH 7, washed with H_2O and eluted with MeOH (19). Because of the similar, strong characteristics between Oasis MCX and the two ExtraBond sorbents, the initial protocol for the ExtraBond sorbents was the same as for the Oasis MCX sorbents. To comparatively evaluate the four sorbents, we performed three extractions for each using pooled urine spiked at 50 ng/mL with the DOAs under study.

The two Oasis sorbents were compared first since they both have polymeric phases and were from the same manufacturer (though one was weak and the other was strong). For greater clarity, the results are expressed as the mean $\%R_{app}$ values from the various DOA families. Specifically, the drugs were divided into opioids, cathinones, ATS, BZD and other DOAs. For Oasis WCX the results were 31%, 46%, 19%, 11% and 19%, respectively, while for Oasis MCX they were 47%, 82%, 78%, 26% and 69%, respectively. Better results were obtained for Oasis MCX. This strong sorbent was compared with a sorbent from a different manufacturer but with the same strong retention polymeric phase (ExtraBond ECX). Since the $\%R_{app}$ values obtained for ExtraBond ECX were the worst (19%, 35%, 29%, 14% and 37% for opioids, cathinones, ATS, BZD and other DOAs, respectively), this sorbent was discarded. Finally, another strong sorbent with a different phase based on silica (ExtraBond SCX) was evaluated and compared with the sorbent that provided the best results (i.e. Oasis MCX). Results for these two sorbents were similar. For ExtraBond SCX, they were 44%, 85%, 77%, 17% and 77%, respectively, for the above drug families. For this reason, both sorbents

were chosen for further studies. All results obtained with the four sorbents are shown in **Figure 1** and, as mentioned earlier, the drugs are divided into five families. For both sorbents, the lowest $\%R_{app}$ values were obtained for opioids (47% and 44%) and especially for BZDs (26% and 17%) except for two of them, i.e. lorazepam (40% and 35%) and bromazepam (38% and 40%). This different behaviour for BZDs could be due to the differences between the pK_a values of these drugs and those of other compounds. A possible explanation for the low $\%R_{app}$ values obtained for the opioids could be their lower retention in the SPE sorbent, which is related to their different chemical structure compared to the other DOAs under study. To increase the low $\%R_{app}$ achieved for some compounds, we tested how the pH of the sample affected urine samples in order to increase their retention strength with the Oasis MCX and ExtraBond SCX sorbents. Specifically, pH values between 2 and 6 were tested for these sorbents by adjusting the sample pH with HCOOH ≥98%. Using different pH values showed that the results were best with ExtraBond SCX and Oasis MCX at pH 4 in both cases. This pH increased the $\%R_{app}$ of some compounds to 20% and 28% for Oasis MCX and ExtraBond SCX, respectively. For example, MOR (opioid) improved its $\%R_{app}$ at pH 4 from 21% to 41% and from 23% to 51% for Oasis MCX and ExtraBond SCX, respectively. With BZDs, the $\%R_{app}$ of alprazolam increased from 11% to 21% and from 11% to 20% for Oasis MCX and ExtraBond SCX, respectively, while the $\%R_{app}$ of diazepam increased from 8% to

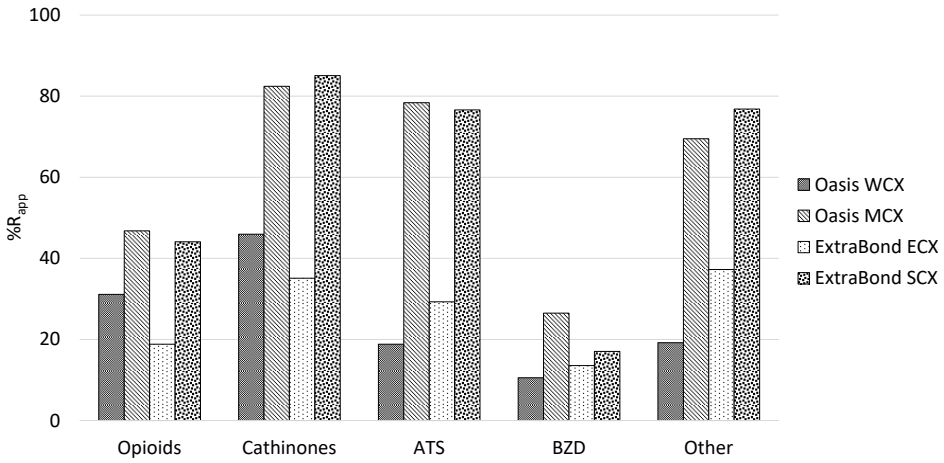

Figure 1. Recovery values for the SPE process using Oasis WCX, Oasis MCX, ExtraBond ECX and ExtraBond SCX of a urine sample spiked at 50 ng/mL.

16% and 9% to 18%, respectively.

Since the commercial ExtraBond SCX sorbent comprised 1000 mg of silica phase and the Oasis MCX comprised 150 mg of polymeric phase, different amounts of Oasis MCX sorbent were tested to evaluate whether the $\%R_{app}$ values increased with a greater amount of sorbent. Specifically, we tested 150 mg, 500 mg and 1000 mg. Our results showed that 500 and 1000 mg of Oasis MCX did not improve the $\%R_{app}$ values even when the elution was performed with 4 mL of elution solvent. Therefore, by comparing the results obtained with 150 mg of Oasis MCX to those obtained with 1000 mg of ExtraBond SCX at pH 4, we found that, for most compounds, the results were similar for both sorbents. However, ExtraBond SCX improved the $\%R_{app}$ of some compounds with very low $\%R_{app}$ in comparison with Oasis MCX, such as KET (other DOAs), BZE (other DOAs), HER (opioid) and lorazepam (BZD). For example, KET had a $\%R_{app}$ of 7% with Oasis MCX and a $\%R_{app}$ of 44% with ExtraBond SCX, while BZE had a $\%R_{app}$ of 28% with Oasis MCX and a $\%R_{app}$ of 71% with ExtraBond SCX. On the other hand, fentanyl (opioid) and norketamine (other DOAs) improved their $\%R_{app}$ with Oasis MCX (from 32% to 47% and from 60% to 78%, respectively). As with Oasis MCX, and to improve the $\%R_{app}$, the amount of ExtraBond SCX sorbent was also studied to ascertain whether increasing the amount to 1500 and 2000 mg achieved better retention of the compounds. Since we studied the amount of sorbent, we also evaluated the elution volume and, since the previous tests were performed with 2 mL of elution solvent, we also studied two further elutions of 1 mL (i.e. 2 mL + 1 mL + 1 mL) for 1000 mg, 1500 mg and 2000 mg. We concluded that, while 2 mL of elution solvent was needed for 1000 mg, 3 mL was needed for 1500 mg and 4 mL was needed for 2000 mg. Better $\%R_{app}$ values were obtained with 1500 mg of sorbent (mean $\%R_{app}$ values were 39%, 72%, 67%, 26% and 63% for the opioids, cathinones, ATS, BZD and other DOAs respectively). 1500 mg was chosen as the optimal amount of sorbent since this generally achieved the best $\%R_{app}$, with the best results being obtained for BZD. This amount also improved some of the lowest $\%R_{app}$ values obtained with the other sorbents without decreasing the others. For example, COD had better results with 1000 mg, followed by 1500 mg and 2000 mg, while methadone had better results with 2000 mg, followed by 1500 mg and 1000 mg.

The optimal SPE procedure was therefore to use 1500 mg of ExtraBond SCX sorbent under the following conditions: 5 mL MeOH, 5 mL H_2O pH 4, 5 mL urine:H_2O pH 4 (50:50, v/v), 2 mL MeOH and 3 mL 5% NH_4OH in MeOH. Then, 100 µL of 1% HCl in MeOH was added before evaporating to dryness with the MiVac. This was finally reconstituted with 500 µL of the initial

mobile phase, filtered through a 0.45 µm PTFE syringe filter, and injected into the LC-MS/MS. Finally, this optimal procedure was tested at a high level of concentration (65 ng/mL) in urine. **Figure 2** shows the $\%R_{app}$ values of the 40 DOAs and metabolites under study. As we explained earlier, the $\%R_{app}$ were lowest with some opioids (HER and fentanyl) and some BZD (alprazolam and diazepam) because of their different chemical structures and different pK_a values, which make their retention strength weaker than that of the other compounds.

Method validation

The instrumental linearity of the method used in this study ranged from the IQL to 125, 200 or 250 ng/mL depending on the DOA. IDLs ranged from 0.01 to 1.5 ng/mL and IQLs ranged from 0.1 to 5.0 ng/mL. To evaluate possible endogenous or exogenous interferences, we analysed drug-free urine samples from laboratory staff members. The exogenous interferences studied were several cannabinoids and some sex-related drugs. No interferences were observed at the same retention times of the DOAs under study.

The method validation parameters are given in **Table II**, which shows that the calibration model was adjusted to one or two matrix-matched calibration curves from the MQL to 75, 100 or 125 ng/mL depending on the DOA under study, with r^2 above 0.99 for all compounds. The MDLs of the method ranged from 0.003 to 0.500 ng/mL, while the MQLs ranged from 0.050 to 1.500 ng/mL. $\%R_{app}$, ME, repeatability and reproducibility were studied at

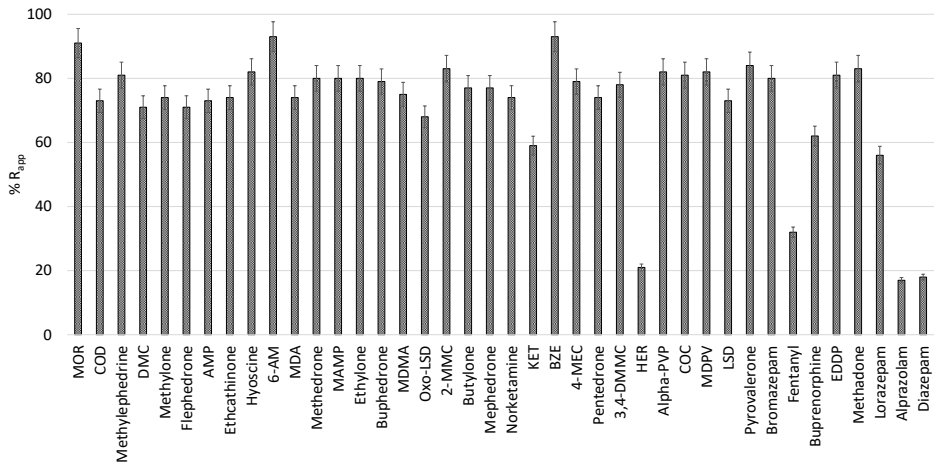

Figure 2. Recovery values of the 40 selected DOAs under the optimal SPE ExtraBond SCX conditions of a urine sample spiked at 65 ng/mL.

Table II. Validation parameters of the DOAs under study.

	Compound	MDL (ng/mL)	MQL (ng/mL)	Linear range (ng/mL)	2 ng/mL		20 ng/mL		65 ng/mL	
					$\%R_{app}$	%ME	$\%R_{app}$	%ME	$\%R_{app}$	%ME
1	MOR	0.050	0.250	0.250 - 75	60	-30	83	-21	91	-10
2	COD	0.025	0.250	0.250 - 125	77	-22	68	-31	73	-25
3	Methylephedrine	0.015	0.250	0.250 - 75	69	-24	72	-32	81	-19
4	DMC	0.025	1.000	1.000 - 75	71	-27	53	-25	71	-29
5	Methylone	0.025	0.250	0.250 - 100	61	-34	68	-30	74	-25
6	Flephedrone	0.050	0.250	0.250 – 5 5 - 100	61	-26	64	-26	71	-19
7	AMP	0.025	0.250	0.250 – 5 5 - 125	65	-26	65	-26	73	-21
8	Ethcathinone	0.015	0.075	0.075 - 100	68	-24	67	-24	74	-22
9	Hyoscine	0.030	0.100	0.100 -100	69	-28	68	-24	82	-19
10	6-AM	0.025	0.250	0.250 – 5 5 - 100	89	-14	83	-16	93	-10
11	MDA	0.050	0.250	0.250 – 5 5 - 100	70	-24	70	-25	74	-22
12	Methedrone	0.050	0.250	0.250 – 5 5 - 100	70	-25	75	-26	80	-18
13	MAMP	0.010	0.050	0.050 – 5 5 - 100	77	-23	73	-18	80	-22
14	Ethylone	0.030	0.100	0.100 – 5 5 - 100	74	-17	72	-16	80	-17
15	Buphedrone	0.025	0.250	0.250 – 5 5 - 100	79	-23	75	-25	79	-24
16	MDMA	0.035	0.100	0.100 – 5 5 -100	73	-22	71	-21	75	-20
17	Oxo-LSD	0.500	1.500	1.500 - 100	42	-31	58	-28	68	-27
18	2-MMC	0.010	0.050	0.050 – 5 5 - 100	78	-16	79	-17	83	-15
19	Butylone	0.050	0.250	0.250 – 5 5 - 100	77	-18	72	-19	77	-15
20	Mephedrone	0.010	0.050	0.050 – 5 5 - 100	78	-21	71	-20	77	-21
21	Norketamine	0.050	0.250	0.250 – 5 5 - 100	59	-29	61	-30	74	-29
22	KET	0.010	0.050	0.050 – 5 5 - 100	75	-26	54	-30	59	-23
23	BZE	0.003	0.050	0.050 – 5 5 - 100	79	-12	89	-11	93	-14
24	4-MEC	0.003	0.250	0.250 – 5 5 - 100	57	-23	76	-23	79	-25
25	Pentedrone	0.010	0.050	0.050 – 5 5 - 100	69	-25	68	-26	74	-26
26	3,4-DMMC	0.010	0.050	0.050 - 75	70	-20	65	-23	78	-18
27	HER	0.100	0.500	0.500 - 125	18	-45	21	-42	21	-38
28	Alpha-PVP	0.003	0.050	0.050 – 5 5 - 100	82	-11	76	-14	82	-10
29	COC	0.010	0.050	0.050 – 5 5 - 75	86	-14	80	-15	81	-14
30	MDPV	0.010	0.050	0.050 – 5 5 - 75	85	-17	77	-15	82	-13
31	LSD	0.010	0.050	0.05 – 5 5 - 100	72	-22	71	-21	73	-20
32	Pyrovalerone	0.010	0.050	0.05 – 5 5 - 100	82	-15	71	-13	84	-12
33	Bromazepam	0.250	0.750	0.75 - 100	83	-23	75	-18	80	-24
34	Fentanyl	0.003	0.050	0.05 – 5 5 - 100	36	-15	24	-18	32	-16
35	Buprenorphine	0.010	0.075	0.075 - 100	47	-62	49	-49	62	-37
36	EDDP	0.003	0.050	0.05 – 5 5 - 100	77	-26	71	-28	81	-19
37	Methadone	0.010	0.050	0.05 – 5 5 - 100	77	-21	77	-17	83	-19
38	Lorazepam	0.050	0.250	0.25 – 5 5 - 100	57	-21	49	-18	56	-22
39	Alprazolam	0.250	1.000	1.00 - 75	19	-25	19	-19	17	-22
40	Diazepam	0.100	0.750	0.75 - 75	15	-23	15	-19	18	-22

three levels of concentration in urine: low (2 ng/mL), medium (20 ng/mL) and high (65 ng/mL). $\%R_{app}$ values ranged from 42% to 89% (2 ng/mL), from 49% to 89% (20 ng/mL) and from 56% to 93% (65 ng/mL) for these three levels. For %ME, these values ranged from -62% to -11%, from -49% to -11% and from -37% to -10%, for low, medium and high concentration levels, respectively. For repeatability and reproducibility, values below 20% were achieved for all three concentration levels. The stability of the DOAs was also evaluated at 10°C for 50 h. Any evidence of degradation was observed during this period with RSD values below 8% for the three calibration levels by injecting each calibrator every 10 h, as it was previously tested by Glickberg et al. (33)

for some synthetic cathinones in urine.

Finally, the method was applied to urine blind samples spiked at three concentrations, i.e., 7 ng/mL, 45 ng/mL and 60 ng/mL. Accuracy was calculated as the error between the concentration calculated with the matrix-matched calibration curve and the concentration spiked in the same sample. The values obtained ranged from 8% to 17% for 7 ng/mL, from 9% to 15% for 45 ng/mL and from 5% to 11% for 60 ng/mL.

This method achieves low MDLs and MQLs and determines the above compounds at the usual levels at which they are present in the urine of drug users. Since it includes some of the classic DOAs that are most consumed, as well as some of the new synthetic drugs currently available on the market and some of their metabolites, it is a useful method for toxicological and forensic laboratories.

Application to real cases

Twenty-two urine samples from women following a detoxification program were analysed using the present method. Of these samples, 91% were found to be positive for one or more DOAs (20 out of 22 samples). COC, BZE, HER, and diazepam were the substances most detected. Detailed results for each sample analysed are shown in **Table III**: COC was found in 16 specimens, its metabolite BZE was found in 19, diazepam was found in 7, lorazepam was found in 4, HER was found in 7, MOR and 6-AM were found in 1, bromazepam was found in 1, MDMA and MDA were found in 1 and fentanyl was found in 1. Multiple drug abuse was identified in 14 samples, mainly involving the consumption of COC as well as some BZDs, which were the second most common group found in urine samples, followed by HER. Once the samples were analysed, the mean concentration for each DOA was calculated (n=3). Then, the uncertainty of each compound was determined with 95% degree of confidence.

Concentrations of COC ranged from <MQL to 535 ng/mL while the concentration of BZE ranged from 0.32 to 9572 ng/mL. The concentration of HER ranged from <MQL to 58.3 ng/mL while MOR and 6-AM were found at 1.78 and 9.4 ng/mL, respectively. The concentrations of lorazepam and diazepam in urine ranged from 0.531 to 13.71 ng/mL and from 2.21 to 144 ng/mL, respectively, while bromazepam was found at 3.74 ng/mL. Finally, MDMA and MDA were found in specimen 18 at 1.52 ng/mL and <MQL, respectively. When the concentrations were found to be outside the linearity range, the samples were diluted to interpolate the results in the matrix-matched calibration curves and the dilution factor was applied to obtain the real concentrations in the urine samples. An example of a chromatogram of sample 13, in which MOR, 6-AM, BZE, COC, HER

and diazepam were identified, is shown in **Figure 3**.

Table III. Compounds Detected in Urine Samples from Women Starting a Detoxification Program (n=3).

Specimen	Analyte detected	Concentration found (ng/mL)	BZE/COC
1	Lorazepam	0.531±0.020	-
	Diazepam	144±3	
2	COC	2.11±0.06	7.4
	BZE	15.6±0.4	
	HER	2.64±0.06	
3	COC	0.48±0.06	37.9
	BZE	18.2±0.4	
4	COC	<MQL	-
	BZE	7.64±0.11	
5	BZE	4.56±0.07	-
	Lorazepam	4.73±0.03	
6	COC	4.90±0.09	406.7
	BZE	1993±17	
7	COC	2.97±0.07	11.1
	BZE	54.1±0.4	
8	COC	<MQL	-
	BZE	0.39±0.05	
	Lorazepam	13.71±0.08	
	Diazepam	5.3±0.3	
9	-	-	-
10	COC	7.72±0.14	1.1
	BZE	8.8±0.1	
11	COC	<MQL	-
	BZE	42.4±0.4	
12	COC	119±2	2.2
	BZE	257.8±0.4	
13	Diazepam	2.21±0.09	37.4
	HER	58.3±0.8	
	MOR	1.78±0.11	
	6-AM	9.4±0.3	
	COC	6.34±0.10	
	BZE	235.5±0.4	
	Diazepam	10.3±0.3	
14	BZE	1.69±0.04	-
	HER	<MQL	
	Bromazepam	3.74±0.14	
15	COC	1.94±0.11	57.9
	BZE	112.4±0.4	
	HER	6.5±0.3	
16	COC	1.31±0.09	36.8
	BZE	47.8±0.4	
	HER	30.9±0.8	
	Lorazepam	3.03±0.03	
17	COC	10.9±0.3	23.7
	BZE	257.9±0.4	
	Diazepam	2.8±0.2	
18	COC	535±2	17.9
	BZE	9572±1	
	HER	6.5±0.3	
	MDMA	1.52±0.03	
	MDA	<MQL	
19	BZE	22.2±0.4	-
	HER	6.5±0.3	
	Diazepam	12.1±0.4	
20	COC	<MQL	-
	BZE	24.4±0.4	
	Diazepam	15.1±0.5	
21	COC	14.5±0.6	4.2
	BZE	60.4±0.4	
	Fentanyl	42.2±0.7	
22	-	-	-

Table III also shows the ratios between the concentrations of BZE and COC. Although these ratios depend on the metabolism of each individual and the time the drug was consumed, low ratios generally mean that COC is not or is hardly metabolised to BZE (recent drug intake), while high ratios indicate that it is strongly metabolised (long-term drug intake). For example, specimen 3 has the highest BZE/COC ratio, which means that most of the COC has been metabolised to BZE and tentatively indicates that consumption was long-term. On the other hand, specimen 10 has the lowest ratio, which may indicate recent drug intake since COC has not been completely metabolised to BZE. In the case of specimen 5, for example, the BZE/COC ratio could not be calculated as COC could not be quantified, perhaps because consumption occurred a long time ago.

Conclusions

A method using SPE followed by LC-MS/MS to determine a group of DOAs (both traditional and new) in urine has been successfully developed. This method performed excellently for the simultaneous screening of 40 DOAs comprising several families and some metabolites. The ability to simultaneously determine a large number of drugs enables high throughput screening and considerably reduces analysis runtimes. Method

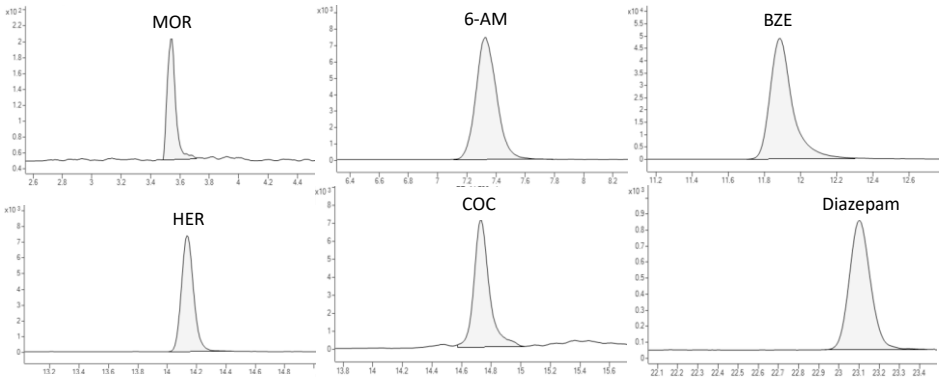

Figure 3. Chromatogram of the analyzed Specimen 13.

optimisation in which four SPE sorbents were tested and compared for all compounds found that the best results were obtained with ExtraBond SCX. This is the first time that this commercial sorbent has been used for extracting a large number of DOAs from urine. The resulting method is capable of achieving very low MDLs and MQLs and is suitable for identifying these compounds in the urine of drug users. To prove applicability of the method, we used it to successfully analyse authentic urine samples from women following a detoxification program. We found that COC was the most consumed drug, and it was confirmed since BZE was also found. This method has therefore proved suitable for toxicological and forensic analysis and for monitoring individuals under a detoxification programme. It has also demonstrated the importance of simultaneously identifying and quantifying multiple DOAs since it can discriminate between various DOAs in toxicological analyses assessing polyconsumption.

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Compliance with ethical standards

Conflict of interest

There are no financial or other relations that could lead to a conflict of interest.

Ethical approval

All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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UNIVERSITAT ROVIRA I VIRGILI
DETERMINATION OF DRUGS OF ABUSE IN URINE AND ORAL FLUID SAMPLES
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3.3.2. Home-made pipette tip solid-phase extraction for the simultaneous determination of 40 drugs of abuse in urine by liquid chromatography-tandem mass spectrometry

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Abstract

Pipette tip solid-phase extraction facilitates the handling of low-volume samples and organic solvents in order to achieve more environmentally-friendly pre-treatment sample techniques. The use of pipette tip extraction was examined for the quick and simple determination of a heterogeneous group of 40 drugs of abuse and some of their metabolites in urine by liquid chromatography coupled to tandem mass spectrometry. Several parameters were studied and optimised, including those which can affect extraction efficiency, such as the amount of sorbent and the volumes and number of aspirating/dispensing cycles of the sample and organic solvents. The linear range of this method was between the quantification limit and 75 or 100 ng mL-1. Detection limits between 0.025 and 0.500 ng mL-1 and quantification limits from 0.100 to 1.500 ng mL-1 were achieved, which are adequate to determine the studied compounds in urine from drug users. Finally, in order to prove its suitability in toxicological and forensic analyses, the method was successfully applied to 22 urine specimens from women who were starting a detoxification program. Cocaine was the most frequently detected substance, as its presence or the presence of its main metabolite was found in 86% of the analysed samples.

Keywords: *Pipette-tip soli-phase extraction · LC-MS/MS · Human urine · Toxicological Analysis · Drugs of abuse*

1. Introduction

Drugs of abuse (DOAs) are a matter of concern due to their high impact on society. They are highly popular substances and each year consumption levels increase further. According to the 2021 World Drug Report, 1 in 18 people from 15 to 64 years old took drugs at least once [1]. In the last ten years, the market has experienced a diversification in the increasing number of DOAs as different synthetic drugs and non-medical pharmaceuticals have grown in number. Apart from established drugs such as cocaine (COC), cannabis, amphetamine-type substances (ATS) or opioids, in recent years new psychoactive substances (NPS) have also come to represent an important group which includes different synthetically-modified established drugs such as synthetic cannabinoids, synthetic cathinones or synthetic opioids. Hundreds of NPS have become highly popular because they mimic the effects of established drugs and in addition, they are easily obtained through different websites at lower prices compared to other DOAs. Moreover, new substances of this type are continually emerging in an attempt to avoid new legislation, so legal control is difficult [1–3]. Therefore, it is important to monitor not only established drugs but also these new NPS which are constantly appearing on the illegal market.

The determination of DOAs in toxicological and forensic analyses has gained more importance in recent years, and as such there is growing interest in developing multiresidue methodologies able to determine these compounds in biological matrices at low levels of concentration (ng mL^{-1}) [4–7]. Urine is one of the most frequently used biological matrices in this field because it offers a window of detection from minutes to days or weeks, which allows both parent compounds and their metabolites to be detected, from low levels of ng mL^{-1} to higher levels. Moreover, this biological sample type has other advantages such as the high volumes of urine that can be collected, in addition to the fact that no specialized personnel is needed to carry this out [8–12]. However, urine is a complex matrix and it can contain different compounds that may interfere with the analytes of interest. For this reason, a pre-treatment step is usually performed in order to clean the sample and extract the compounds of interest. Even though there are some well-established extraction techniques such as liquid-liquid extraction (LLE) and solid phase extraction (SPE), which have been commonly used in different methodologies previously, the recent trend is to miniaturize the extraction for the purpose of increasing sample throughput and reducing sample and solvent consumption, thus highlighting its benefits in the context of green

chemistry [13–15]. In this regard, different strategies derived from conventional SPE such as pipette tip SPE (PT-SPE), also known as disposable pipette extraction (DPX), have been employed, among others [15–18]. The PT-SPE is based on a μ-SPE in which the sorbent is placed in a tip between two filters of frites or cotton. Its main advantages are that it is easy to operate and low that it requires low volumes of sample, organic solvents and sorbent. However, it also presents some drawbacks such as the scarcity of articles in this field and the limited availability of commercial sorbents in tip format, so as a result some practitioners opt for home-made tips [15]. Some of the most important parameters in PT-SPE optimization are the type and amount of sorbent, and the volumes and cycles of loading, washing and elution. There are two options for increasing the volume of sample loading, washing and elution: by increasing the volume of the pipette, or by performing more cycles in the same step. Increasing the loading volume or cycle means increasing the pre-concentration factor [15,18–22].

PT-SPE has become an effective tool for the purification and precon-centration of different analytes in various matrices, as various authors have demonstrated. This technique has been employed in the determination of DOAs in biological matrices [18,20–33] and in some cases, it has been used in urine samples [24,25,27,29–31]. For example, Shi et al. [27] determined a group of four ATS in urine by gas chromatography (GC) coupled to tandem mass spectrometry (MS/MS) and in the pre-treatment, they used home-made PT based on a 3D ionic liquid ferrite functionalized graphene oxide nanocomposite sorbent. In the loading step, they performed at least ten aspirating/dispensing cycles to extract the analytes: two washing cycles and five eluting cycles. With this protocol, they achieved recovery values higher than 89% for the four ATS with limits of quantification (LOQs) between 8.4 and 27.5 ng mL^{-1}. Ellison et al. [24] developed a method for the determination of 28 DOAs in urine by GC-MS and they performed an extraction of the analytes with a commercial PT made of sulfonated functional groups on a divinyl benzene sorbent. They loaded the sample in one cycle and equilibrated it for 20 seconds before washing it in two cycles and finally, they eluted the analytes using two organic solvents, one for acidic drugs and one for neutral drugs. Recoveries obtained ranged from 57 to 133% with limits of detection (LODs) between 1.04 and 74.0 ng mL^{-1}. Montesano et al. [29] also performed a PT-SPE pre-treatment for the determination of cannabinoids and their metabolites in urine by liquid chromatography (LC) MS/MS. For the

extraction, they used commercial OMIX C_{18} tips from Agilent and their protocol consisted of five aspirating/dispensing cycles of the sample, three cycles of washing and five cycles of eluting the analytes. They achieved recoveries of between 65 and 85% with LODs and LOQs from 2 to 4 and 6 to 10 ng mL^{-1}, respectively. Kumazawa et al. [30] also used a C_{18} tip for the PT-SPE procedure in the determination of amphetamine (AMP) and methamphetamine (MAMP) in urine. The extraction was performed by 25 aspirating/dispensing cycles of the sample, washing with one cycle and eluting with five cycles. They obtained recovery values of between 78 and 88% and LODs of 0.08 and 0.10 ng mL^{-1} and LOQs of 0.50 ng mL^{-1}.

As can be observed, different types of sorbents have been employed for the PT-SPE technique with very promising results. According to the previous experience of our research group, when both weak and strong mixed-mode cationic exchange sorbents were used for the same DOAs, excellent results were achieved. In particular, ExtraBond SCX sorbent was previously studied and proved effective for the extraction of different types of DOAs from urine but using conventional SPE and LC-MS/MS [34].

The present paper details a method based on a home-made PT-SPE followed by LC-MS/MS for the determination of a large group of DOAs and their metabolites in urine using a commercial mixed-mode SPE sorbent, ExtraBond SCX. Parameters such as the amount of sorbent and the volume and cycles of the loading, washing and elution steps are optimized. Moreover, the developed method is applied to urine specimens from women who are starting a detoxification program. The results are also compared to the results obtained with conventional SPE pre-treatment to confirm this methodology as a reliable tool for use in toxicological and forensic analyses. As far as we know, this is the first time to date that the ExtraBond SCX sorbent has been used for determining a large group of DOAs and their metabolites in urine using a home-made PT-SPE.

2. Experimental

2.1. Standards and materials

Drug standards were purchased from LGC Standards (Luckenwalde, Germany) and Sigma Aldrich (St. Louis, MO, USA). N-ethylcathinone (eth-cathinone), 4-fluoromethcathinone (flephedrone), 3,4-methylenedioxy-N-ethylcathinone (ethylone), buphedrone, 4-methylmethcathinone (mephedrone), 2-methylmethcathinone (2-MMC), 3,4-methylenedioxymethcathinone (methy-lone), butylone, 4-methylethcathinone (4-MEC), beta-ethylmethcathinone (pentedrone), 3,4-dimethylmethylca-thinone (3,4-DMMC), alpha-pyrrolidino-valerophenone (alpha-PVP), methylene-

dioxypyrovalerone (MDPV), 4-methxymethcathinone (methedrone), dimethylcathinone, pyrovalerone, methamphetamine (MAMP), amphetamine (AMP), 3,4.methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), morphine (MOR), 6-acetylmorphine (6-AM), cocaine (COC), benzoylecgonine (BZE), fentanyl, methadone, buprenorphine, heroin (HER), codeine (COD), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), diazepam, alprazolam, lorazepam, bromazepam, lysergic acid diethylamide (LSD), 2-oxo-3-hydroxy-LSD (oxo-LSD), ketamine (KET), norketamine, 4-methylephedrine and hyoscine.

Stock solutions were prepared individually in methanol (MeOH) at concentrations of 100 mg L^{-1}, 1000 mg L^{-1}, and 2000 mg L^{-1}, depending on the compound, and frozen at -20°C. A mixture of these was also prepared in MeOH at 2 mg L^{-1} and kept in the freezer. Diluted working solutions were then prepared in water (H_2O) for injection.

Acetonitrile (ACN) and H_2O for LC-MS were purchased from Scharlab (Barcelona, Spain). MeOH was obtained from J.T. Baker (Deventer, The Netherlands). Hydrochloric acid (HCl) ≥37%, formic acid (HCOOH) ≥98%, and ammonium hydroxide (NH_4OH) ≥28% were obtained from Sigma-Aldrich (St. Louis, MO, United States). Ultrapure water was purchased from a water purification system (Merck Millipore, Darmstadt, Germany).

ExtraBond SCX (1000 mg/6mL) sorbents were purchased by Scharlab. A Transferpette® S micropipette from Brand (Wertheim, Germany) was used for the extraction. For evaporating the final solutions, a MiVac Duo sample concentrator from Genevac (Ipswich, UK) was used.

2.2. LC-MS/MS conditions

An Agilent model 1200 series LC coupled with an Agilent 6460 series triple quadrupole mass spectrometer with an electrospray ionisation (ESI) interface from Agilent Technologies (Waldbronn, Germany) was used in the present study. For instrumental control and data analysis the Agilent MassHunter Workstation Software version B.09.00 was used.

The LC-MS/MS conditions were the same as in the previous study in which the same group of drugs were determined in urine using SPE as pre-treatment [34]. A Luna Omega 5 µm Polar C_{18} (150 mm x 4.6 mm, 5 µm) column from Phenomenex (Torrance, CA, United States) with a Security Guard from Phenomenex was used for the chromatographic separation. An injection volume of 10 µL with a flow rate of 0.6 mL min^{-1} of the mobile phase was employed. The mobile phase was constituted by A: 0.1% HCOOH in H_2O

and B: 0.1% HCOOH in ACN in gradient mode. The gradient started at 15% B and was maintained for 5 minutes. It was increased to 35% in 7 minutes, to 80% in 13 minutes, and then to 100% in 1 minute. It was held for 2 minutes before returning to the initial conditions in 1 minute and finally maintained for 5 minutes.

The optimization of the MS/MS parameters was performed by injecting each compound at 1 mg/L in a mixture of H_2O:MeOH (50:50, v/v). A Multiple Reaction Monitoring (MRM) mode in positive polarity with ten different windows was employed for the acquisition. For this, the two most intensive transitions between the parent ion and the product ions were selected. The optimal MS/MS parameters were: capillary voltage, 2500 V; gas temperature, 350 ºC; gas flow rate, 13 L min^{-1}; and nebulizer pressure, 30 psi. The fragmentor was set between 50 and 125 V, and the collision energy (CE) between 4 and 42 eV (Table 1).

2.3. Preparation of home-made PT-SPE tips

The homemade PT-SPE tips were prepared by using two pipette tips of 200 µL and 1000 µL, respectively, ExtraBond SCX sorbent and two small pieces of cotton. First, to construct the PT device, a small piece of cotton was placed inside the 200 µL tip and it was tightened. 10 mg of ExtraBond SCX sorbent were then introduced into the tip and finally, another small piece of cotton was inserted. These three layers were tightened to prevent sorbent loss both above and below the tip. Finally, the 1000 µL tip was inserted into the 200 µL and used with the 1000 µL Transferpette® S micropipette.

2.4. Urine collection and PT-SPE pre-treatment

Urine was collected using polypropylene tubes and was kept in the freezer at -20 °C. Urine samples were then thawed and 250 µL of urine was mixed with 250 µL of ultrapure H2O at pH 4 (adjusted with HCOOH ≥98%). For the PT-SPE, the sorbent was conditioned and activated using one aspirating /dispensing cycle of 500 µL of MeOH and 500 µL of ultrapure H_2O at pH 4, respectively. One cycle of 250 µL of the urine mixture: H_2O at pH 4 (50:50, v/v) was then aspirated and dispensed, washed with one cycle of 500 µL of MeOH and eluted with one cycle of 1000 µL of 5% NH_4OH in MeOH. After the PT-SPE process, 100 µL of 1% HCl ≥37% in MeOH was added to the final solution. The extracts were evaporated to dryness using a MiVac sample concentrator, reconstituted with 250 µL of mobile phase at initial conditions and filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter before the analysis.

Table 1. MRM parameters of the DOAs under study.

	Compound	Rt (min)	Precursor ion	Product ion 1	CE 1 (eV)	Product ion 2	CE 2 (eV)	Product ion 3	CE 3 (eV)	Fragmentor voltage (V)
1	MOR	3.70	3.70	286.1	165	40	153	40	157	40
2	COD	6.28	6.28	300.2	165	40	199	36	153	42
3	Methylephedrine	6.67	6.67	180.0	162	8	147	10	131	10
4	DMC	6.86	6.86	178.1	105	18	133	14	72	18
5	Methylone	7.26	7.26	208.0	160	16	132	20	190	10
6	Flephedrone	7.32	7.32	182.1	164	15	149	15	123	15
7	AMP	7.41	7.41	136.1	119	4	91	10	-	-
8	Ethcathinone	7.49	7.49	178.1	160	12	132	14	105	18
9	Hyoscine	7.86	7.86	304.0	156	20	138	20	121	20
10	6-AM	8.49	8.49	328.2	211	36	193	36	165	40
11	MDA	8.51	8.51	180.1	163	6	135	10	133	10
12	Methedrone	8.98	8.98	194.1	176	10	161	16	145	16
13	MAMP	9.02	9.02	150.1	119	8	91	10	-	-
14	Ethylone	9.15	9.15	222.1	174	18	204	12	146	18
15	Buphedrone	9.39	9.39	178.1	91	15	131	15	132	15
16	MDMA	10.20	10.20	194.1	163	10	133	15	105	15
17	Oxo-LSD	10.70	10.70	356.0	237	20	222	20	265	12
18	2-MMC	11.00	11.00	178.1	160	8	145	15	119	15
19	Butylone	11.15	11.15	222.1	174	14	204	10	191	10
20	Mephedrone	11.39	11.39	178.1	160	8	145	15	119	15
21	Norketamine	11.68	11.68	224.0	207	8	179	12	125	12
22	KET	12.42	12.42	238.0	125	20	220	12	207	12
23	BZE	12.76	12.76	290.1	168	20	105	20	82	20
24	4-MEC	12.79	12.79	192.1	174	10	146	16	145	18
25	Pentedrone	13.33	13.33	192.1	174	10	132	16	91	18
26	3,4-DMMC	14.36	14.36	192.1	174	10	159	15	133	15
27	HER	14.75	14.75	370.0	211	36	268	36	165	42
28	Alpha-PVP	15.10	15.10	232.2	91	22	105	25	126	25
29	COC	15.95	15.95	304.2	182	18	105	22	82	22
30	MDPV	15.70	15.70	276.2	126	25	135	25	175	20
31	LSD	16.34	16.34	324.2	231	24	208	26	180	26
32	Pyrovalerone	16.56	16.56	246.2	105	22	126	26	119	26
33	Fentanyl	17.44	17.44	337.2	188	22	105	24	-	-
34	Bromazepam	18.57	18.57	316.0	182	30	209	30	288	30
35	Buprenorphine	19.29	19.29	468.0	55	48	396	42	414	35
36	EDDP	20.18	20.18	278.0	234	30	249	20	186	30
37	Methadone	20.78	20.78	310.2	265	12	105	18	-	-
38	Lorazepam	21.15	21.15	321.0	275	20	303	10	230	20
39	Alprazolam	21.35	21.35	308.4	281	24	274	24	204	30
40	Diazepam	22.03	22.03	285.0	193	30	154	30	222	30

2.5. Validation

The European guidelines for workplace drug testing in urine were followed for the method validation [35]. The validated parameters were: sensitivity, selectivity, linearity, instrumental and method detection and quantification limits (IDLs and IQLs and MDLs and MQLs, respectively), matrix effect (ME), apparent recoveries (R_{app}), precision as reproducibility (inter-day) and repeatability (intra-day), stability and accuracy. Moreover, tolerances for retention times and ion ratios of ±2.5% and ±20% were considered [36].

The instrumental linearity was evaluated by using different working solutions of drugs with neat standards between 0.01 and 250 ng mL^{-1} considering the determination coeffi-

cient (r^2). IDLs were considered the lowest detectable point with a signal-to-noise (S/N) ratio ≥3 and IQLs as the lowest concentration in the calibration curve with S/N ≥10.

The method linearity was studied by using matrix-matched calibration curves with a mixture of drugs in urine at concentrations of between 0.01 and 125 ng mL^{-1}. The method linearity, MDLs and MQLs criteria were considered the same as for the instrumental ones, but for extracted samples. The R_{app}, ME, repeatability and reproducibility were evaluated at three concentration levels: 2 ng mL^{-1} (low), 20 ng mL^{-1} (medium) and 65 ng mL^{-1} (high) by using five replicates (n=5). Reproducibility (n=5 during five days) and repeatability (n=5 in the same day) were evaluated as relative standard deviation (%RSD).

The parameters of selectivity and specificity were studied considering the possible endogenous and exogenous interferences from urine. The stability was studied for 50 hours at 10 °C by analysis replicates of 2, 20 and 65 ng mL^{-1} in urine (n=5). The accuracy was evaluated by analysing three blind samples which had been spiked by a member of the laboratory staff before all experimental procedures had been carried out, and calculating the error between the obtained concentration and the real one.

22 urine samples from anonymous women starting a drug detoxification programme at the Centre Català de la Solidaritat (CECAS), in Tarragona, Spain, were analysed using the methodology developed. Urine samples were collected in polypropylene tubes at the moment of admission and frozen at -20°C prior to the PT-SPE sample pre-treatment.

3. Results and discussion

3.1. PT-SPE optimization

As previously mentioned in the Introduction section, several authors have used the PT-SPE strategy to extract different compounds from urine [18,21]. In the different reported strategies, several parameters have been demonstrated to affect extraction efficiency and for that reason, the present research examines the influence of these parameters in order to achieve the best extraction efficiency for the DOAs under study. The parameters evaluated were the amount of sorbent, the volumes and the aspirating/dispensing cycles of the loading, washing and elution steps and the reusability of the tips.

A general scheme of the PT-SPE procedure is shown in Fig. 1. The extraction procedure is the same as in conventional SPE but in the case of PT-SPE, the solvents are not percolated through the sorbent, but instead are aspirated and dispensed. The term aspirate refers to filling up the pipette with the volume indicated while

dispensing means emptying the pipette of the volume aspirated. This combination is known as one aspirating/dispensing cycle. This action can be performed several times and thus several aspirating/dispensing cycles can be carried out. In this sense, a volume is aspirated, then it is dispensed to waste, again another volume is aspirated and dispensed and this action is performed a fixed number of times (cycles). In the same way as for conventional SPE, the first step of pre-treatment is conditioning the sorbent, the second step is loading the sample, the next step is washing and the final step is eluting the analytes.

The starting conditions for optimization were based on a previous study in which the same group of DOAs were determined in urine by SPE and LC-MS/MS [34]. These conditions were adapted with slight modifications to the PT-SPE methodology and were as follows: 1 cycle of 1 mL MeOH and 1 cycle of 1 mL H_2O pH 4 adjusted with HCOOH ≥98% (conditioning), 1 cycle of loading 1 mL of a mixture of urine:H_2O pH 4 (50:50, v/v) (loading), 1 cycle of 1 mL MeOH (washing) and 1 cycle of 1 mL 5% NH_4OH in MeOH (elution). 100 µL of 1% HCl in MeOH were added to the methanolic solution. It was then evaporated to dryness with the MiVac and finally, reconstituted with 250 µL of initial mobile phase and filtered through a 0.45 µm PTFE filter prior to its injection in the LC-MS/MS. A drug-free urine sample spiked at 50 ng mL^{-1} containing the DOAs under study was used for this purpose and each analysis was performed in triplicate.

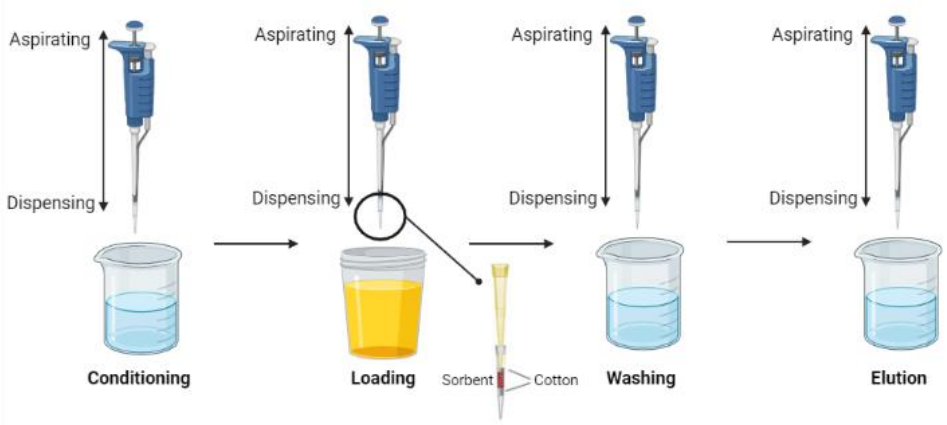

Figure 1. A scheme of the PT-SPE procedure.

Amount of sorbent. Based on the previous study in which different sorbents were tested and compared for the SPE of the same analytes in urine, ExtraBond SCX was selected for the present PT-SPE strategy [34]. The amount of this sorbent was evaluated and 5, 10, 15, 20, 25 and 30 mg were all considered suitable for the 200 µL tip. Problems with blocking and operational difficulty were observed when 25 and 30 mg were used. The results for the other tested conditions can be seen in Fig. 2, in which the mean area of all the studied compounds is represented for 5, 10, 15 and 20 mg of sorbent. As can be observed in Fig. 2, the results showed that the adsorption ability of the sorbent increased by increasing the amount of sorbent to 10 mg, and for this amount of sorbent, the maximum peak area values were obtained and then decreased. This suggests that 5 mg of sorbent was not enough for this sample volume while amounts higher than 10 mg probably need more volume to elute the analytes and thus the areas were reduced. In general, the areas obtained with the optimal amount were around 8% higher compared to 5 mg, around 11% higher compared to 15 mg and around 32% higher compared to 20 mg. Therefore 10 mg of ExtraBond SCX sorbent was chosen as the optimal amount.

Sample loading volume. With 10 mg of sorbent, the sample loading volume was evaluated and for this purpose 200, 250, 500 and 1000 µL of urine:H_2O pH 4 (50:50, v/v) were studied. Even though a low $\%R_{app}$ was achieved with all the tested volumes, as Fig. 3 shows, the one which provided the best results was 250 µL. To better illustrate this, the DOAs in the figure are divided into five different families: opioids, cathinones, ATS, BZD and other DOAs and the mean %Rapp of each family is shown. In the case of 250 µL of loading volume, $\%R_{app}$ of 38, 22, 31, 19 and 37% were obtained respectively for the aforementioned families. The figure also shows that the %Rapp increases to 250 µL and then, for higher volumes, lower $\%R_{app}$ were obtained. The lowest $\%R_{app}$ values were obtained for BZD. This can be explained by the difference between their pK_a (around 2 for some of them, except for lorazepam and bromazepam) compared to the pK_a values of most of the DOAs under study (between 7 and 10). Therefore, as was also observed in the previous SPE strategy [34], at a pH of 4, some BZD are not charged and they are only retained by reversed-phase interactions, while the other compounds are also retained by ionic interactions. However, at a pH lower than 4, the $\%R_{app}$ of the other families decreased and thus pH 4 was used for the further procedure.

Aspirating/dispensing cycles of sample loading volume. The number of aspirating/dispensing cycles of loading volume is of great importance in the PT-SPE technique since the adsorption of

Figure 2. Effect of mg of sorbent on the area obtained by the analytes under study.

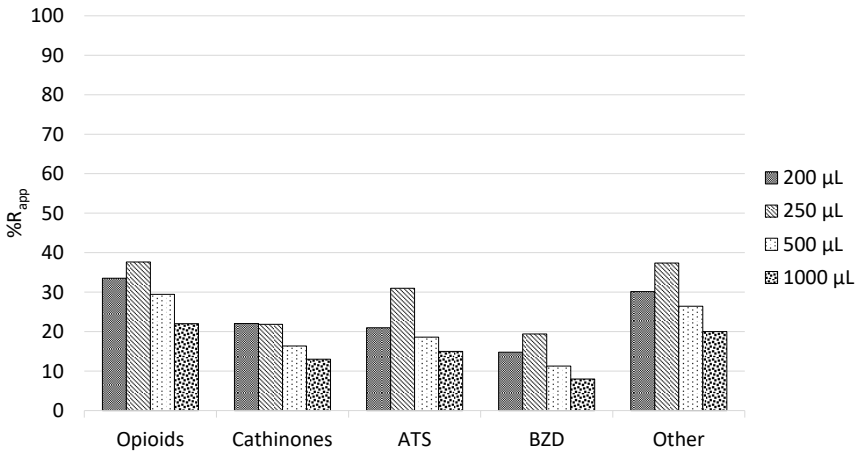

Figure 3. Evaluation of different loading volumes of urine:H_2O at pH 4 (50:50, v/v) at a spiked concentration of 50 ng mL^{-1}.

the analytes may vary after some cycles and there is no agreement about this issue in the literature, as was mentioned in the Introduction [24,25,27,29–31]. For this reason, one, two and three aspirating/dispensing cycles of the sample loading volume were evaluated. Fig. 4 shows that one cycle achieved the best results for all families except for opioids, which achieved slightly better $\%R_{app}$ with two cycles (4% more). However, as the general trend was a

decrease in the $\%R_{app}$ as the aspirating/dispensing cycles increased, one cycle was found to be the optimal number. This could be because the retention strength was not sufficient to retain the analytes with more than one cycle or because after several cycles, the elution volume was not sufficient to elute all the analytes. Therefore increasing the number of cycles, which means increasing the volume, led to lower retention, as was also observed in the study of the loading sample volume. Moreover, if two cycles of 250 µL are compared to one cycle of 500 µL, similar results can be observed except in the case of opioids, where two cycles of 250 achieved better $\%R_{app}$.

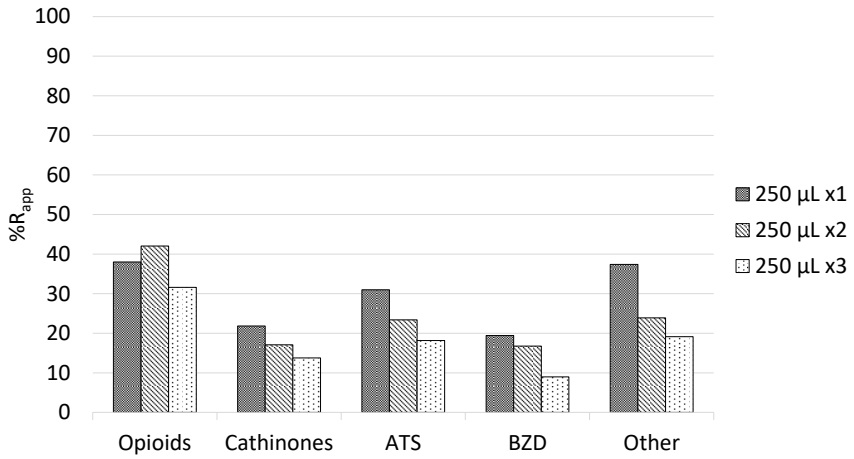

Figure 4. Evaluation of different aspirating/dispensing loading cycles of urine:H_2O at pH 4 (50:50, v/v) at a spiked concentration of 50 ng mL^{-1}.

Washing volume and cycles. The washing volume was also studied and 250, 500 and 1000 µL of MeOH were tested. The general trend between these volumes was that lower $\%R_{app}$ was obtained for 250 µL whereas higher and similar values were obtained for 500 and 1000 µL. As these two volumes achieved similar $\%R_{app}$, and as a lower volume of organic solvent was used with 500 µL, this was selected washing as the volume. In particular, values of 39, 34, 38, 26 and 43% were obtained for opioids, cathinones, ATS, BZD and other DOAs. It was observed that the $\%R_{app}$ of BZD increased by decreasing the washing volume. In particular, using 250 µL of MeOH, BZD achieved $\%R_{app}$ of 32%, maybe because, as mentioned above, they are not retained as much as other DOAs due to their pK_a value. However, as the $\%R_{app}$ of the other families decreased with 250 µL, 500 µL of MeOH was considered the optimal volume. In

addition, the washing cycles were also studied and one, two and three cycles of 500 µL of MeOH were evaluated. One washing cycle achieved the best results compared to two and three cycles, maybe because the retention strength decreased when the cycles were increased. In these cases, two cycles achieved similar or slightly lower %Rapp for opioids and cathinones (37 and 34%, respectively) but lower $\%R_{app}$ for ATS, BZD and other DOAs (30, 15 and 33%, respectively). If two cycles of 500 µL are compared to one cycle of 1000 µL, similar results were obtained for most families except for ATS, which was 8% higher for one cycle of 1000 µL. Therefore one cycle of 500 µL of MeOH was finally selected.

Elution volume and cycles. 500 and 1000 µL of 5% NH_4OH in MeOH were the initial elution volumes studied. It was observed that the best results in terms of $\%R_{app}$ were achieved with 1000 µL of elution solvent compared to 500 µL (between 1 and 17% higher). As 1000 µL was the maximum volume of the micropipette used, more cycles were evaluated to see if the $\%R_{app}$ could be increased. In particular, the combination of one cycle of 1000 µL with one cycle of 500 µL (total volume of 1500 µL) and two cycles of 1000 µL (total volume 2000 µL) were studied. Results showed similar results of $\%R_{app}$ for these two combinations and both of them were similar to one cycle of 1000 µL (up to 5% more for the total volumes of 1500 µL and 2000 µL). Considering the similar $\%R_{app}$ achieved and that the extraction time with one cycle is shorter than when two cycles are combined, one cycle of 1000 µL of 5% NH_4OH in MeOH was selected as the optimal elution step.

Reusability. The possibility of reusing the tips to save costs was also investigated by analysing five samples with the same tip and five samples with different tips. The deviation in terms of %RSD of the $\%R_{app}$ achieved was compared in both cases. It was observed that higher %RSD were obtained by reusing the same tip (between 14 and 27%) than by using new ones (between 8 and 20%). One possible explanation is that after the first use, the particles of sorbent move and they are not as compressed between the two pieces of cotton as in the first use and thus, the retention is different than when they are first pressed. Therefore, due to the higher changes when the tip was reutilised and the low cost of a new tip, tips were not reutilised in order to avoid wider variation in the results.

Table 2 shows a summary of the extraction conditions used for the PT-SPE procedure. After the extraction, 1% HCl in MeOH was added to the methanolic solution prior to its evaporation to dryness with the MiVac. It was reconstituted with 250 µL of mobile phase at initial conditions, filtered through a 0.45 µm PTFE syringe

Table 2. Extraction conditions of the PT-SPE procedure for the determination of 40 DOAs in urine by LC-MS/MS.

Parameter	Optimum condition
Type of sorbent	ExtraBond SCX
Amount of sorbent	10 mg
Conditioning	1 cycle of 500 µL of MeOH and 1 cycle of 500 µL of H_2O at pH 4
Loading sample volume and cycles	1 cycle of 250 µL of the mixture urine: H_2O at pH 4 (50:50, v/v)
Washing volume and cycles	1 cycle of 500 µL of MeOH
Elution volume and cycles	1 cycle of 1000 µL of 5% NH_4OH in MeOH

filter, and finally analysed with the LC-MS/MS.

3.2. Method validation

The instrumental linearity of the present method was between the IQL and 125, 200 or 250 ng mL^{-1} depending on the DOA. The method achieved IDLs from 0.01 to 1.5 ng mL^{-1} and IQLs between 0.1 and 5.0 ng mL^{-1} for the DOAs under study. To evaluate the possible endogenous or exogenous interferences, different drug-free urine samples from laboratory staff members were analysed. In particular, the exogenous interferences studied involved some benzodiazepines and some sex-related drugs. The results showed that no interferences could be observed at the same retention times of the DOAs under study. The validation parameters are shown in Table 3.

The calibration model was adjusted to one or two matrix-matched calibration curves from the MQL to 75 or 100 ng mL^{-1}, depending on the DOA, all with r^2 higher than 0.990. The MDLs of the present method were between 0.025 to 0.500 ng mL^{-1}, while the MQLs were between 0.100 and 1.500 ng mL^{-1}. The $\%R_{app}$, ME, repeatability and reproducibility were studied at low (2 ng mL^{-1}), medium (20 ng mL^{-1}) and high (65 ng mL^{-1}) levels of concentration. In the case of $\%R_{app}$, values were between 11 and 55% (2 ng mL^{-1}), from 12 to 58% (20 ng mL^{-1}) and from 16 to 62% (65 ng mL^{-1}). %ME achieved values between -18 and -50%, -12 and -48% and between -11 and -47%, for 2, 20 and 65 ng mL^{-1}, respectively. For reproducibility and repeatability, values lower than 20% were obtained for the three levels of concentration. Stability was evaluated for 50 hours at 10 °C by analysis replicates of 2, 20 and 65 ng mL^{-1} in urine (n=5) without evidence of degradation in this period. %RSD values lower than 7% for the mentioned calibration levels were obtained by injecting each calibrator sample every

Table 3. Validation parameters of the DOAs under study

	Compound	MDL (ng/mL)	MQL (ng/mL)	Linear range (ng/mL)	2 ng/mL		20 ng/mL		65 ng/mL	
					$\%R_{app}$	%ME	$\%R_{app}$	%ME	$\%R_{app}$	%ME
1	MOR	0.050	0.250	0.250 - 5 5 - 100	55	-50	55	-42	54	-47
2	COD	0.050	0.250	0.250 - 5 5 - 75	42	-41	58	-36	53	-27
3	Methylephedrine	0.500	1.500	1.500 - 75	40	-34	42	-35	47	-34
4	DMC	0.250	1.000	1.000 - 75	15	-22	12	-24	16	-21
5	Methylone	0.050	0.250	0.250 - 5 5 - 75	45	-31	45	-27	52	-24
6	Flephedrone	0.250	1.000	1.000 - 75	19	-46	21	-48	19	-45
7	AMP	0.250	1.000	1.000 - 75	25	-33	31	-27	29	-26
8	Ethcathinone	0.250	1.000	1.000 - 75	29	-24	33	-19	36	-22
9	Hyoscine	0.150	1.000	1.000 - 75	46	-18	48	-14	51	-15
10	6-AM	0.250	0.750	0.750 - 75	43	-38	53	-34	56	-24
11	MDA	0.250	1.000	1.000 - 75	34	-24	36	-25	42	-22
12	MAMP	0.250	0.750	0.750 - 5 5 - 100	32	-33	31	-26	36	-16
13	Methedrone	0.100	0.500	0.500 - 75	45	-28	47	-26	52	-18
14	Ethylone	0.250	0.750	0.750 - 100	48	-32	45	-29	49	-21
15	Buphedrone	0.250	0.750	0.750 - 75	22	-33	26	-30	29	-21
16	MDMA	0.100	0.500	0.500 - 5 5 - 100	41	-29	39	-24	48	-19
17	Oxo-LSD	0.500	1.500	1.500 - 75	17	-26	35	-22	43	-18
18	2-MMC	0.250	1.000	1.000 - 75	18	-31	22	-26	26	-23
19	Butylone	0.250	0.750	0.750 - 100	38	-25	37	-22	48	-20
20	Mephedrone	0.100	0.500	0.500 - 5 5 - 100	33	-35	38	-32	45	-25
21	Norketamine	0.250	1.000	1.000 - 100	20	-31	29	-27	35	-25
22	KET	0.250	1.000	1.000 - 100	35	-32	38	-24	43	-20
23	BZE	0.075	0.250	0.250 - 5 5 - 100	37	-33	48	-21	51	-21
24	4-MEC	0.075	0.250	0.250 - 5 5 - 100	27	-21	32	-20	41	-18
25	Pentedrone	0.100	0.500	0.500 - 5 5 - 100	20	-33	25	-27	35	-21
26	3,4-DMMC	0.100	0.500	0.500 - 5 5 - 100	36	-36	37	-35	42	-23
27	HER	0.100	0.500	0.500 - 5 5 - 75	12	-33	14	-28	17	-26
28	Alpha-PVP	0.025	0.100	0.100 - 5 5 - 75	29	-34	33	-30	41	-22
29	COC	0.030	0.100	0.100 - 5 5 - 100	52	-19	58	-12	62	-11
30	MDPV	0.030	0.100	0.100 - 5 5 - 100	47	-25	50	-22	54	-16
31	LSD	0.030	0.100	0.100 - 5 5 - 100	39	-29	45	-27	54	-19
32	Pyrovalerone	0.030	0.100	0.100 - 5 5 - 75	31	-19	33	-17	36	-11
33	Fentanyl	0.100	0.500	0.500 - 5 5 - 100	49	-22	51	-22	53	-17
34	Bromazepam	0.500	1.500	1.500 - 75	54	-32	58	-26	61	-22
35	Buprenorphine	0.500	1.500	1.500 - 75	34	-29	36	-27	38	-25
36	EDDP	0.075	0.250	0.250 - 5 5 - 100	40	-29	44	-25	49	-21
37	Methadone	0.150	0.500	0.500 - 5 5 - 100	30	-28	34	-26	41	-17
38	Lorazepam	0.500	1.500	1.500 - 100	11	-32	19	-28	21	-18
39	Alprazolam	0.500	1.500	1.500 - 100	47	-26	53	-24	57	-21
40	Diazepam	0.025	0.100	0.100 - 5 5 - 100	46	-19	47	-18	53	-16

10 h. The method was then tested with blind urine samples spiked at three different concentrations by a laboratory staff member. They were spiked at 6 ng mL^{-1}, 32 ng mL^{-1} and 58 ng mL^{-1} in urine and the accuracy was calculated as the error between the concentration calculated with the matrix-matched calibration curve and the concentration spiked in the same sample. These errors were between 9 and 18% for 6 ng mL^{-1}, from 9 to 17% for 32 ng mL-1 and from 3 to 14% for 58 ng mL^{-1}.

Even though the MDLs and MQLs are not as low as with the previously conventional SPE strategy developed for the same group [34], the present PT-SPE methodology achieved the usual levels at which these compounds can be found in urine samples from drug abusers by means of a simple, quick, green technique with a high throughput.

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3.3. PT-SPE LC-MS/MS application to real cases

With drug rehabilitation programmes, it is important to be able to provide useful methods for controlling patient admissions and monitoring throughout the programme. As Table 4 shows, the PT-SPE technique developed was applied to twenty-two urine specimens from women starting a detoxification programme and the results were compared to the results obtained by the conventional SPE methodology [34]. A total of 20 positive samples out of 22 analysed were confirmed, finding COC, BZE, HER, diazepam, lorazepam, bromazepam, MDMA, MDA, MOR, 6-AM and fentanyl. As Table 4 shows, different compounds were found, which can be attributed to the poly-consumption of DOAs. This could be detected in 14 specimens and most of them were due to the consumption of COC with a BZD and/or HER. All concentrations out of the linear range were diluted for their correct quantification. COC was the most frequently detected substance due to its presence in 16 specimens and to the presence of its main metabolite BZE in 19, which could be found in some specimens where COC could not be detected. Concentrations of COC were between <MQL and 540 ng mL^{-1}, while for BZE this ranged from <MQL to 9585 ng mL^{-1}. The BZD diazepam, lorazepam and bromazepam were found in 7, 4 and 1 specimens, respectively. HER was found in 7 samples while other compounds such as MOR, 6-AM, MDMA, MDA and fentanyl were detected in 1 specimen. An example of specimen 16 is shown in Fig. 5, in which COC, BZE, HER and lorazepam were found.

Table 4 also compare the results of the present study with those obtained using the previously developed SPE methodology [34]. As can be observed, variations lower than 10% in most cases and up to 18% were achieved between the two methodologies. However, some compounds could not be quantified due to the higher MQLs achieved in the present methodology compared to the previous SPE. This is the case with lorazepam in specimen 1 and BZE in specimen 8. Even so, the PT-SPE methodology has proven its reliability and suitability for determining a large number of DOAs and their metabolites in urine and can achieve similar results to a conventional SPE procedure but with a more environmentally-friendly procedure.

4. Conclusions

A new strategy based on home-made PT-SPE for the simultaneous determination of 40 DOAs and some of their metabolites has been successfully developed. This is the first time that ExtraBond SCX sorbent has been used for the self-assembly PT-SPE pre-

Table 4. Concentration of the DOAs detected in urine samples form women starting a detoxification program and comparison with the SPE methodology.

Specimen	Analyte detected	Concentration found with the PT-SPE method (ng mL^{-1})	Concentration found with the SPE method (ng mL^{-1}) [34]
1	Lorazepam	<MQL	0.531±0.020
	Diazepam	137±4	144±3
2	COC	2.45±0.05	2.11±0.06
	BZE	16.2±0.5	15.6±0.4
	HER	3.0±0.1	2.64±0.06
3	COC	0.45±0.05	0.48±0.06
	BZE	17.8±0.5	18.2±0.4
4	COC	<MQL	<MQL
	BZE	7.5±0.5	7.64±0.11
5	BZE	4.4±0.1	4.56±0.07
	Lorazepam	4.91±0.08	4.73±0.03
6	COC	5.2±0.3	4.90±0.09
	BZE	1970±22	1993±17
7	COC	2.92±0.05	2.97±0.07
	BZE	58±1	54.1±0.4
8	COC	<MQL	<MQL
	BZE	<MQL	0.39±0.05
	Lorazepam	14.1±0.3	13.71±0.08
	Diazepam	5.3±0.2	5.3±0.3
9	-	-	-
10	COC	7.6±0.3	7.72±0.14
	BZE	9.2±0.4	8.8±0.1
11	COC	<MQL	<MQL
	BZE	43.2±0.8	42.4±0.4
12	COC	122±4	119±2
	BZE	262±5	257.8±0.4
	Diazepam	2.45±0.15	2.21±0.09
13	HER	57±1	58.3±0.8
	MOR	2.1±0.3	1.78±0.11
	6-AM	9.9±0.7	9.4±0.3
	COC	6.0±0.3	6.34±0.10
	BZE	232±4	235.5±0.4
14	Diazepam	9.8±0.6	10.3±0.3
	BZE	1.8±0.1	1.69±0.04
	HER	<MQL	<MQL
	Bromazepam	3.4±0.3	3.74±0.14
15	COC	1.87±0.18	1.94±0.11
	BZE	111.8±0.9	112.4±0.4
	HER	6.1±0.6	6.5±0.3
16	COC	1.4±0.1	1.31±0.09
	BZE	49±1	47.8±0.4
	HER	33±2	30.9±0.8
	Lorazepam	3.3±0.4	3.03±0.03
17	COC	10.7±0.3	10.9±0.3
	BZE	260±4	257.9±0.4
	Diazepam	2.6±0.4	2.8±0.2
18	COC	540±5	535±2
	BZE	9585±24	9572±1
	HER	5.9±0.6	6.5±0.3
	MDMA	1.5±0.1	1.52±0.03
	MDA	<MQL	<MQL
19	BZE	23.1±0.8	22.2±0.4
	HER	6.0±0.7	6.5±0.3
	Diazepam	12.0±0.6	12.1±0.4
20	COC	<MQL	<MQL
	BZE	24.5±0.4	24.4±0.4
	Diazepam	14.7±0.7	15.1±0.5
21	COC	14.7±0.7	14.5±0.6
	BZE	61±1	60.4±0.4
	Fentanyl	40±1	42.2±0.7
22	-	-	-

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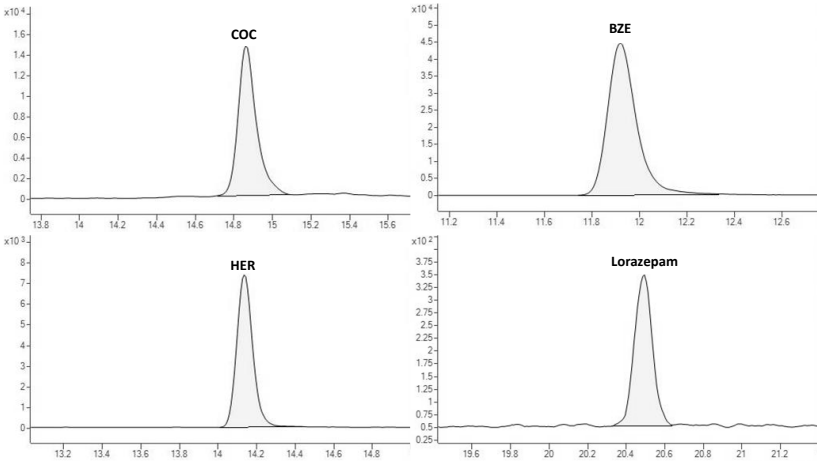

Figure 5. Chromatogram of the analysed specimen 16.

treatment for the extraction of a large group of drugs in a complex biological sample such as urine. The PT-SPE technique offers an easy, fast and environmentally friendly pre-treatment for extracting a large group of substances from urine. Even though weaker sensitivity is achieved in the present study compared to other conventional SPE strategies, the technique is able to determine these compounds at low levels of ng mL^{-1} in urine samples, as was proven when the method developed was applied to real urine samples from women starting a detoxification programme. In these samples, COC was the most frequently detected substance, its metabolite BZE being found in most of them and the polyconsumption of different DOAs was also observed, mainly due to the presence of COC with a BZD. Providing multiresidue methods which are able to determine different types of DOAs as well as their metabolites is highly significant, since some of them can be totally metabolised and can only be found due to the presence of their metabolites.

In the future, more methodologies based on the PT-SPE strategy should be developed for determining different types of DOAs as they offer easy, cheap, green pre-treatments. Moreover, different sorbents should be tested to evaluate their suitability with large groups of drugs and if possible, the proposed technique should be applied to more DOAs to expand its applicability.

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Compliance with ethical standards

Conflict of interest

There are no financial or other relations that could lead to a conflict of interest.

Ethical approval

All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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3.3.3. Discussion of results

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The most important results from the methods developed to determine 40 DOAs and their metabolites using SPE and PT-SPE in urine by LC-MS/MS are discussed below.

Four mixed-mode cationic exchange sorbents have been evaluated and compared for a large group of DOAs and their metabolites. In particular, Oasis WCX, Oasis MCX, ExtraBond ECX and ExtraBond SCX have been the selected SPE cartridges [1]. Different pH values between 2 and 6 have been studied to achieve the best extraction efficiencies for all the compounds, being ExtraBond SCX the one that provided better values at pH 4. However, the lowest recoveries were obtained by some BZD as their pK_a values were much lower than the other drugs and thus, at pH 4 they were not as retained as them. Even though, satisfactory recoveries between 42 and 93% were obtained for most of the compounds studied. Moreover, high sensitivity has been also achieved and LOQs from 0.050 to 1.500 ng mL^{-1} have also been obtained.

In an attempt to achieve a green strategy able to determine several DOAs and their metabolites in urine, a home-made PT-SPE device has been developed for the first time in our research group. As different mixed-mode cationic exchange sorbents were evaluated in the previous study, ExtraBond SCX, which was the optimal, was used as extracting sorbent in the PT-SPE device. This was easily prepared by placing the sorbent between two pieces of cotton, which were tightened inside a pipette tip. In comparison with other methodologies that performed various aspirating/dispensing cycles of sample loading to achieve a higher preconcentration, the present study performed only one cycle and obtained similar LODs and LOQs than the mentioned with a faster method [2–5]. Even that the recoveries and sensitivity values were not as good as the ones in the previous study performing a conventional SPE, the concentration levels achieved are suitable to detect the compounds studied in urine from drug abusers.

Finally, to prove the applicability of the developed methodologies, both of them were applied to 22 urine specimens from women starting a detoxification program in CECAS. The results showed that 20 out of 22 samples were positive in at least one drug and 14 of the were positive in more than one. The most detected substance was COC, which was found in 16 samples and its metabolite BZE in 19.

It has been observed that in the previous year of pandemic there has been an increase in the drug consumption and in particular, of BZD because of the sadness of the population [6]. This has been proved by the analysis of the CECAS samples, which showed that BZD were the second most consumed drug family, finding 12 BZD among all the 22 specimens. This shows the importance of including the metabolites in the study, because the detection of the metabolite is longer than the parent drug. The results obtained of the samples analysed from the two developed methods were also compared. Even that in some cases the PT-SPE strategy was not able to quantify some compounds because they were present at very low levels of concentration, similar results were obtained in both cases, showing that they can be a useful tool for toxicological and forensic analyses.

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CHAPTER 4. CONCLUSIONS

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The most important conclusions obtained from the results achieved during the present Doctoral Thesis are detailed below:

- Different methodologies for drug determination in biological samples have been successfully developed. All of them achieved low levels of ng mL^{-1}, which are suitable to detect these compounds in biological samples from drug users.

- Both LC-HRMS and LC-MS/MS based methods have been developed for the determination of a group of synthetic cathinones in urine and OF samples. In particular, LC-MS/MS using QqQ as analyser has demonstrated to provide higher sensitivity than LC-HRMS.

- An OF sample pre-treatment has been carried out in the same collector device using very low volumes of organic solvent. The Salivette® device allows the clean-up of the sample without the need of any additional pre-treatments.

- A CE-MS/MS method has been successfully developed to enantiomerically determine three ATS and their metabolites in urine. Among the six different chiral selectors evaluated in the coupling of CE-MS/MS using the CMT approach, the one that provided the best enantioresolution was 0.2% of sulphated γ-CD.

- The use of mixed-mode cationic exchange sorbents has provided, in general, excellent extraction efficiencies for different DOAs in urine samples. Apart from the high preconcentration that it can be achieved, an exhaustive cleaning of the matrix is also performed, which means lower ME and thus higher R_{app}.

- In the case of synthetic cathinones and ATS extraction from urine, Oasis MCX and Oasis WCX were compared. Oasis MCX was the selected sorbent for cathinones because lower ME were obtained while for ATS, Oasis WCX was the optimal for its higher R_{app}.

- For the determination of 40 DOAs and metabolites in urine, Oasis, WCX, Oasis MCX, ExtraBond ECX and ExtraBond SCX were evaluated and compared. ExtraBond SCX provided higher extraction efficiencies and less interferences in comparison with the other sorbents. However, some BZD (alprazolam and diazepam) achieved low R_{app} values because of their lower pK_a compared to the other DOAs.

- The PT-SPE strategy developed allows the simultaneous extraction and clean-up of a large group of DOAs by only using a pipette, a tip and low amount of sorbent between two pieces of cotton.

- The use of PT-SPE has provided an easy and green strategy to determine 40 DOAs and metabolites in urine samples by LC-MS/MS.

- In the urine samples from drug abusers from CECAS, polyconsumption has been observed as a common practice. Among all the DOAs found, the most consumed drugs have been COC and BZD, being the first, detected in 19 out of 22 samples between 0.48 and 535 ng mL^{-1}.

APPENDIX

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Appendix I. Abbreviation from this Doctoral Thesis.

2-MMC	2-methylmethcathinone
3,4-DMMC	3,4-dimethylmethylcathinone
4-MEC	4-mehylethylcathinone
6-AM	6-acetylmorphine
ACN	acetonitrile
ADHD	attention-deficit hyperactivity disorder
AMP	amphetamine
ATS	amphetamine-type substances
BGE	background electrolyte
BZD	benzodiazepines
BZE	benzoylecgonine
C6G	codeine-6-glucuronide
CBD	cannabidiol
CBN	cannabinol
CD	cyclodextrin
CE	capillary electrophoresis
CMT	counter-current migration technique
COC	cocaine
COD	codeine
CSA	Controlled Substance Act
DAD	diode-array detector
DCM	dichloromethane
DEA	Drug Enforcement Administration
DLLME	dispersive liquid-liquid microextraction
DMC	dimethylcathinone
DMS	dried matrix spots
DOA	drug of abuse
DOFS	dried oral fluid spots
dSPE	dispersive solid-phase extraction
DUS	dried urine spots
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EKS	electrokinetic supercharging

EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EME	ecgonine methyl ester
EOF	electroosmotic flow
ESI	electrospray ionisation
EU	European Union
EWS	Early Warning Systems
FASI	field-amplified sample injection
FL	fluorescence detector
FPSE	fabric phase sorptive extraction
GBL	Δ-butyrolactone
GC	gas chromatography
GHB	Δ-hydroxybutyrate
HCD	higher-energy collisional dissociation
HCl	hydrochloric acid
HCOOH	formic acid
HER	heroin
HESI	heated electrospray ionization
HMMA	4-hydroxy-3-methoxymethamphetamine
HRMS	high-resolution mass spectrometry
IDL	instrumental detection limit
INCB	International Narcotics Control Board
IPA	2-propanol
IQL	instrumental quantification limit
KET	ketamine
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LSD	lysergic acid diethylamide
LTTM	low transition temperature mixture
M3G	morphine-3-glucuronide
M6G	moprhine-6-glucuronide
MAE	microwave-assisted extraction
MAMP	methamphetamine

MDA	3,4-methylenedioxyamphetamine
MDL	method detection limit
MDMA	3,4-methylenedioxymethamphetamine
MDPV	3,4-methylenedioxypyrovalerone
ME	matrix effect
MeOH	methanol
MEPS	microextraction by packed sorbents
MOR	morphine
MQL	method quantification limit
MRM	Multiple Reaction Monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSPE	magnetic solid-phase extraction
MSTFA	N-Methyl-N-trimethylsilyl-trifluoroacetamide
m_t	migration time
MTD	methadone
NaCl	sodium chloride
NaOH	sodium hydroxide
NPS	new psychoactive substances
OF	oral fluid
OHMA	p-hydroxymethamphetamine
PFPA	pentafluoropropionic anhydride
PFT	partial filling technique
PMA	p-methoxyamphetamine
PMMA	p-methoxymethamphetamine
PTFE	polytetrafluoroethylene
PT-SPE	pipette-tip solid-phase extraction
QqQ	triple quadrupole
QToF	quadrupole time-of-flight
R_{app}	apparent recovery
RSD	relative standard deviation
R_t	retention time
SALLE	salting-out liquid-liquid extraction
SFC	supercritical fluid chromatography
SLE	supported liquid extraction

SPE	solid-phase extraction
SPME	solid-phase microextraction
TCA	trichloroacetic acid
THC	Δ9-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-Δ9-tetrahydrocannabinol
THC-OH	11-hydroxy-Δ9-tetrahydrocannabinol
R_t	retention time
UNODC	United Nations Office on Drugs and Crime
US	United States
US-DLLME	ultrasound-assisted dispersive liquid-liquid microextraction
VAMS	volumetric absorptive microsampling
α-PVP	alpha-pyrrolidinovalerophenone

Appendix II. List of publications.

- S. Pascual-Caro, N. Fontanals, F. Borrull, C. Aguilar, M. Calull, Solid-phase extraction based on cation-exchange sorbents followed by liquid chromatography high-resolution mass spectrometry to determine synthetic cathinones in urine, Forensic Toxicology. 38 (2020) 185–194. doi: 10.1007/s11419-019-00508-8.
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- S. Pascual-Caro, F. Borrull, C. Aguilar, M. Calull, Comparison of different chiral selectors for the enantiomeric determination of amphetamine-type substances in human urine by solid-phase extraction followed by capillary electrophoresis-tandem mass spectrometry, Electrophoresis. 43 (2021) 437–445. doi: 10.1002/elps.202100231.
- S. Pascual-Caro, F. Borrull, C. Aguilar, M. Calull, Development of a Liquid Chromatography–Tandem Mass Spectrometry Method for the Simultaneous Determination of 40 Drugs of Abuse in Human Urine: Application to Real Cases, Journal of Analytical Toxicology. (in press). doi: 10.1093/jat/bkac020.
- S. Pascual-Caro, F. Borrull, M. Calull, C. Aguilar, Recent chromatographic and related based methods for the determination of drugs of abuse in urine and oral fluid: A review from 2018 to June 2021, TrAC – Trends Anal. Chem. (accepted).

- S. Pascual-Caro, F. Borrull, M. Calull, C. Aguilar, Home-made pipette tip solid-phase extraction for the simultaneous determination of 40 drugs of abuse in urine by liquid chromatography-tandem mass spectrometry (submitted).

Appendix III. Communications in scientific congresses

- Oral communication entitled "Determination of synthetic cathinones in urine by solid-phase extraction followed by liquid chromatography-high resolution mass spectrometry" in the *XVIII Scientific meeting of the Spanish Society of Chromatography and Related Techniques* (Granada, Spain).
- Oral communication entitled "Determination of synthetic cathinones in oral fluid by liquid chromatography-tandem mass spectrometry" in the *1st Iberian Meeting in Separation Sciences & Mass Spectrometry* (Santiago de Compostela, Spain).
- Oral communication entitled "Determinació de catinones sintètiques en mostres d'orina i saliva" in the *Onzena Trobada de Joves Investigadors dels Països Catalans* (Vilanova i la Geltrú, Spain).
- Oral communication entitled "Enantiomeric determination of amphetamine, methamphetamine and MDMA in human urine by CE-MS/MS" in the *27th International Symposium on Electrophoretic and Liquid Phase Separation Techniques* (Virtual edition).
- Oral communication entitled "Simultaneous determination of 40 drugs of abuse and some metabolites in human urine by SPE followed by LC-MS/MS" in the *XX Scientific meeting of the Spanish Society of Chromatography and Related Techniques* (Virtual edition).

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