# SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS: DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES

**Tatiana Baciu**

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**Tatiana Baciu**

DOCTORAL THESIS

Supervised by

Dr. Carme Aguilar Anguera and Dr. Marta Calull Blanch

Department of Analytical Chemistry and Organic Chemistry

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2017

La Dra. CARME AGUILAR ANGUERA, Professora Agregada del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili, i

La Dra. MARTA CALULL BLANCH, Professora Titular del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili,

FEM CONSTAR:
Que la present Tesi Doctoral, que porta per títol: “SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS: DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES”, presentada per Tatiana Baciu per optar al grau de Doctor per la Universitat Rovira i Virgili amb menció internacional, ha estat realitzada sota la nostra direcció a l’Àrea de Química Analítica del Departament de Química Analítica i Química Orgànica d’aquesta universitat. Tots els resultats presentats són fruit d’experiències realitzades per l’esmentada doctoranda, i compleix els requeriments per a poder optar a la menció internacional.

I, per a que consti, signem aquest document a Tarragona, 27 de juny de 2017.

Dra. Carme Aguilar Anguera | Dra. Marta Calull Blanch

***To myself*** ☺

Escribir los agradecimientos pone punto y final a esta Tesis Doctoral que es el resultado del esfuerzo conjunto de las directoras, la Dr. Carme Aguilar Anguera y la Dr. Marta Calull Blanch, y mi persona. A lo largo de los últimos cinco años hemos puesto a prueba nuestras capacidades y conocimientos en el desarrollo de nuevos métodos analíticos basados en la electroforesis capilar, cumpliendo todas nuestras expectativas. Por ello, en primer lugar, quisiera agradecer la dedicación de las directoras, ya que sin su ayuda no habría sido posible llevar el presente trabajo a término. ¡Gracias por estar al pie del cañón cada día!

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## ABSTRACT

The (ab)use of drugs of abuse (DOAs) represents important social and health problem worldwide. Detection and quantification of these substances in biological samples of drug (ab)users have important clinical and forensic applications, from diagnosis of acute/lethal intoxication, monitoring of compliance/abstinence in detoxification centres to identification of drugs affecting performance at work (workplace drug testing), while driving (driving under the influence of drugs) and in sports (doping control). Within this context, there is an emerging need for the continuous development of analytical methods suitable to determine these compounds in biological samples.

Capillary electrophoresis (CE), with its high efficiency and resolution, possibility of automation, minimal consumption of reagents and low sample volume for analysis, as well as it low cost, is a well-established analytical separation technique that has been successfully applied for drug testing purposes. However, despite these great benefits, CE suffers from poor concentration sensitivity, especially when ultraviolet detection (UV) is used. To overcome this problem, several strategies have been developed, including the in-line coupling between solid-phase extraction (SPE) and CE (in-line SPE-CE), which has proven to be highly useful for improving the sensitivity of CE in the analysis of different kinds of compounds, including DOAs. Traditionally, in in-line SPE-CE, a hand-made SPE microcartridge containing an appropriate sorbent material is inserted near the injection end of the capillary.

In view of the above, the research included in this Doctoral Thesis aimed to develop inexpensive, green and easily performed methods suitable for determining DOAs in human biological samples by means of in-line SPE-CE-UV. Classic DOAs, such as cocaine, codeine, methadone and morphine, were investigated since these substances are frequently consumed. The major metabolite of cocaine, benzoylecgonine, and a heroin-specific metabolite, 6-acetylmorphine, were also included. Meanwhile, new psychoactive substances, such as mephedrone and 3,4-methylenedioxypyrovalerone, have gained popularity among young people and teenagers in recent years. The abuse of these drugs has soared to reach epidemic levels. For this reason, these compounds, as well as 4-methylephedrine (mephedrone metabolite), were also analysed here. With respect to the biological samples analysed, the research particularly focused on hair and urine. Urine represents one of the most conventional matrices used to detect (ab)use of DOAs, while hair has emerged over the past years as an alternative and/or

complementary matrix due to its extended detection window, allowing retrospective monitoring of consumption for several months and even up to years before sample collection.

The experimental work presented in this Doctoral Thesis has been structured into two parts. In the first part, the potential of in-line SPE-CE-UV was explored for the quantitative determination of different DOAs in hair samples. Three scientific articles, published in different international journals, have resulted from the obtained data here. In all cases, a 2 mm length segment of a capillary of 150 μm internal diameter (i.d.) packed with 60 μm particle size Oasis HLB (reversed-phase) was used for the construction of the in-line SPE-CE microcartridge. Particularly, the first paper deals with the determination of cocaine and benzoylecgonine in hair samples from drug abusers. To extract the analytes from the hair matrix, an overnight acidic digestion procedure was followed from the literature and successfully combined with the in-line SPE-CE system.

Since the enantiomers of a given drug might have different pharmacological and toxicological effects, the development of analytical methods that could cope with the identification and quantification of each of them in biological samples can provide valuable assistance in interpreting drug testing results. Of the DOAs examined in this Doctoral Thesis, methadone occurs as a chiral molecule. In view of this, the second paper deals with the simultaneous determination of 6-acetylmorphine, benzoylecgonine, cocaine, codeine and morphine, all of which are non-chiral compounds, and the enantiomers of methadone, in hair samples from drug abusers. α- and β-cyclodextrin (CD) were evaluated as chiral selectors, of which α-CD proved to be the most appropriate for the intended purpose. To extract the analytes from the hair matrix, a procedure based on pressurized liquid extraction (PLE) was developed and satisfactorily combined with the in-line SPE-CE system. An acidic aqueous solution was used as the extracting medium. Compared to the overnight acidic digestion applied in our previous work (around 16 hours), the use of PLE instrument enabled us to extract the target drugs in a massively reduced time (around 15 minutes), representing an excellent achievement in the field of hair drug testing.

Mephedrone and its metabolite, and 3,4-methylenedioxypyrovalerone also exist as chiral molecules. Within this framework, the third paper focuses on the development of a new method based on the in-line coupling between SPE and CD-modified-CE with a previous sample pretreatment procedure based on PLE for the enantiomeric

determination of these substances in hair samples. α-, β- and γ-CD, and highly sulphated-γ-CD were evaluated as chiral selectors, of which β-CD provided the best results in terms of resolution. The PLE was completed within 8 minutes using a basic aqueous solution as the extracting medium.

The three analytical methods established in the first part were validated in line with the guidelines of the Scientific Working Group for Forensic Toxicology and Society of Toxicological and Forensic Chemistry. Linearity, repeatability, reproducibility, limit of detection and quantification, and relative recoveries were evaluated using pooled drug-free hair samples spiked with the studied analytes. In all cases, the limits of detection (LODs) achieved with UV detection for the hair matrix were in the range of 0.02-0.13 ng/mg, except for morphine (LOD of 1.0 ng/mg), which are generally in accordance with the cut-offs recommended by the Society of Hair Testing, except for morphine. Quantitative precision in both intra-day and day-to-day experiments was very satisfactory with relative standard deviations (RSDs) less than 13%. Relative recoveries greater than 80% were obtained. In addition, the hair pretreatment procedures proposed here based on acidic digestion and PLE allow a considerable simplification of the sample preparation process, especially when compared to procedures involving the use of organic solvents. This is because the hair extract solutions can be directly injected into the in-line SPE-CE system without the need for evaporation and reconstitution steps.

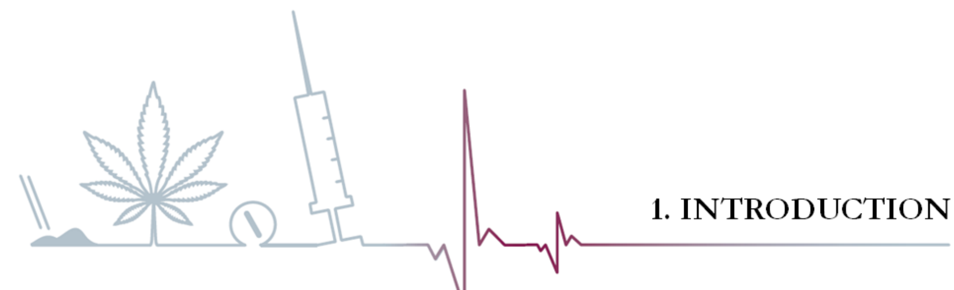
Recently, magnetic particles have emerged as a new generation of adsorbent materials used in sample preparation. Due to their magnetic properties, these can be easily manipulated inside a CE capillary just by the application of a magnetic field. Therefore, magnetic particles represent a very simple way to create an analyte concentrator inside the capillary. Taking advantage of this feature, the second part, which consists of a scientific article reported in an international journal, focuses on the ability to construct SPE microcartridges inside a 50-μm internal diameter CE capillary using magnetic particles as the adsorbents for the extraction of DOAs. Particularly, silica-coated iron oxide magnetic particles functionalized with octadecyl groups were synthesized, loaded into the capillary and used to preconcentrate cocaine, codeine, methadone and morphine in urine samples from drug abusers. For trapping the magnetic particles, two commercial neodymium iron boron magnets were used.

The analytical method established in the second part was also validated in accordance with the guidelines of the Scientific Working Group for Forensic Toxicology and

Society of Toxicological and Forensic Chemistry. Linearity, repeatability, reproducibility, limit of detection and quantification, and relative recoveries were evaluated using pooled drug-free urine samples spiked with the studied analytes. The developed method provided LODs for urine samples in the range 20-50 ng/mL, which are below the cut-offs recommended by the European Workplace Drug Testing Society and Substance Abuse and Mental Health Services Administration. Quantitative precision in both intra-day and day-to-day experiments was satisfactory with RSDs less than 20%. Relative recoveries greater than 76% were obtained.

Combining magnetic particles in-line with CE proved to offer advantages over the conventional in-line SPE-CE configuration used in the first part of the present Doctoral Thesis, resulting in an easier and faster way to create an analyte concentrator inside the capillary, which does not require time-consuming procedures for manufacture. The use of a very simple instrumentation and the achievement of satisfactory sensitivity make this strategy a reasonably attractive analytical tool for routine analysis of DOAs.

The use of both in-line SPE-CE configurations has proven to be valuable and promising approaches to detect DOAs in biological samples. The research results obtained here contribute to the overall knowledge of both techniques in the field of drug testing, proposing cheap, simple and environmentally friendly CE-based methodologies.



# 1. INTRODUCTION

A drug is a natural or synthetic substance that is designed to produce a specific set of psychological or physiological effects on the human body. Most drugs are produced legitimately by drug manufactures and are prescribed for particular illnesses, injuries, or other medical problems. These drugs are most often taken and used for an intended purpose. Sometimes, however, they have effects that people find pleasurable and therefore are taken for purposes other than for which they are prescribed.

Within this context, in addition to legally produced pharmaceutical drugs, there are also substances that have no legitimate, recognized medicinal purposes, but are produced and ingested entirely for their psychoactive effects. Many of these drugs are naturally occurring substances or are extracted or derived from natural substances, usually plants. Others are purely synthetic compounds. Legally produced drugs that are abused and drugs produced for no reason other than abuse are called abused drugs, drugs of abuse, or illicit drugs. Drugs of abuse (DOAs) is the term that will be used in the present Doctoral Thesis.

People start experimenting with DOAs for several reasons, e.g., curiosity, to have a good time, because friends are doing it, or an attempt to improve their athletic performance or ease issues such as stress, worry, anxiety or depression [1].

As humans, we inherently enjoy feeling good. Under normal circumstances, neurons in the brain's reward pathway release a transmitter called dopamine, which controls movement, emotion, perception, motivation and pleasure. Dopamine is then released into the synapse, which results in a jolt of pleasure. When DOAs are used, the amount of dopamine in the synapse is increased, heightening the feeling of pleasure to the point of euphoria. Because the brain associates enjoyment with experiences that help us survive, the brain remembers what caused this feeling and wants to repeat it more [2]. Therefore, when DOAs make us feel good, our brain wants more.

Repeated drug use affects the functions of γ-aminobutyric acid, which is an inhibitory transmitter that prevents our nervous system from being overstimulated [2]. In this sense, the brain never thinks it is satiated, leading to more frequent consumption. Within this framework, the drug that a person takes eventually reaches the level of significance that other physiological needs have.

Most DOAs are addictive. Addiction is a chronic, relapsing disease characterized by compulsive drug seeking and use despite negative consequences, as well as by long-

lasting changes in the brain. People who are addicted have strong cravings for the drug, making it difficult to stop using. Most DOAs alter a person's thinking and judgment, which can increase the risk of injury or death from driving under the influence, overdose or infectious diseases from unsafe sexual practices or needle sharing. During pregnancy, drug use can lead to neonatal abstinence syndrome, a condition in which a baby can suffer from dependence and withdrawal symptoms after birth [3].

Every year, the United Nations Office on Drugs and Crime (UNODC) releases a World Drug Report, which analyses current trends and statistics. As shown in Figure 1, the 2016 edition reveals that one in twenty adults around the world between the ages of 15 and 64 years (quarter of a billion people), which corresponds to five per cent of the global population, used at least one drug of abuse in 2014, and 29 million of them were regular users. In addition, almost 12 million people were injecting drugs, of whom one in seven (two million people) were living with human immunodeficiency virus, one in two (six million people) were infected with hepatitis C, and one in eleven (one million people) were infected with hepatitis B. The annual report also shows that, each year, around 207,000 deaths are related to or associated with the use of DOAs and over half of these deaths are estimated to be cases of fatal overdose [4].

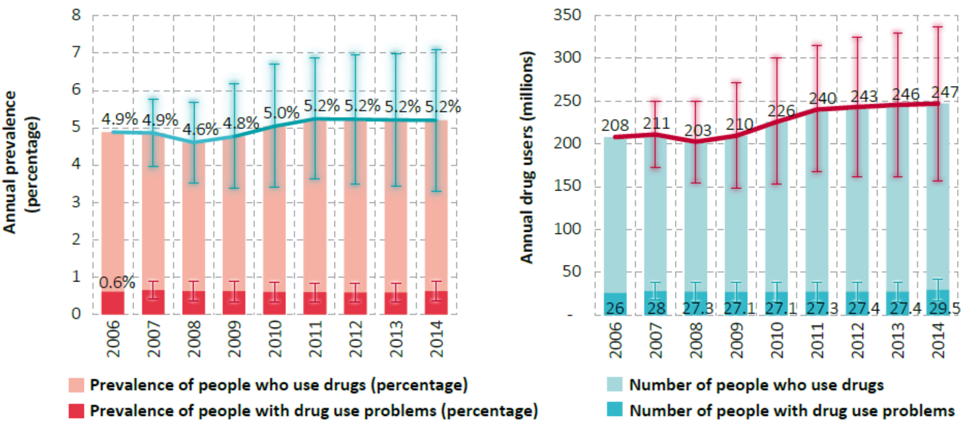



**Figure 1.** Estimated global trends between 2006 and 2014 [4].

Within this context, identification and quantification of DOAs in biological samples can provide useful information on an individual's use and/or exposure to a drug of abuse. Such evidence can be important in workplace testing (reemployment and

random testing), clinical and rehabilitation settings (identification of DOAs associated with overdoses or drug abuse, compliance and/or abstinence monitoring in detoxification treatment centres), forensics and the criminal justice system (cause of death, at the time of arrest, rehabilitation testing of prisoners and offenders on parole), schools and testing of professional athletes (eligibility to compete).

In view of the above, the present Doctoral Thesis focuses on the development of novel analytical methods based on the in-line coupling between solid-phase extraction (SPE) and capillary electrophoresis-ultraviolet detection (CE-UV) suitable for determining DOAs in biological samples. Particularly, methods have been developed for the detection of classic DOAs, such as cocaine, benzoylecgonine, codeine, morphine, 6-acetylmorphine and methadone, as well as new psychoactive substances, such as mephedrone, one of its metabolite and 3,4-methylenedioxypyrovalerone, in hair and urine samples. Firstly, the main characteristics of the most frequently (ab)used DOAs are summarized. This is followed by an overview of the analytical techniques that are currently most widely used for drug testing in biological matrices, especially hair and urine. After the introduction, the objective of the present Doctoral Thesis is laid out. The third chapter presents the results and discussion of the studies derived from the experimental research included in paper format. Finally, the main conclusions that can be drawn from the studies are presented.

## 1.1. DRUGS OF ABUSE

There are a wide variety of DOAs. They range from synthetic to naturally occurring substances and can have a wide variety of psychoactive effects. They occur in a number of forms from plant materials and plant extracts to powders, tablets and capsules, liquids and solids. Some can be easily grown, whereas others are manufactured using sophisticated chemistry.

There are several ways of classifying DOAs. The Federal Comprehensive Drug Abuse Prevention and Control Act of 1970, commonly known as the Controlled Substances Act, places them into one of five schedules based upon their medical use, potential for abuse, and safety or dependence liability [5]. Another way of classifying drugs is by origin: naturally occurring (e.g., marijuana, psilocybin mushrooms), plants extracts (e.g., cocaine, morphine) semi-synthetic (e.g., heroin, lysergic acid diethylamide) and synthetic (e.g., amphetamines, barbiturates) [6]. However, the most popular way of classifying drugs is by their psychoactive effects. Under this scheme, DOAs are separated into four classes: stimulant, depressant, narcotic and hallucinogen (Figure 2). Because of its popularity, this classification system will be discussed in more detail in the following paragraphs. Over the last decade, the traditional drug scene has been supplemented by the quick emergence of hundreds of new psychoactive substances (NPS) [4, 7]. Therefore, this group of drugs is also considered in this Doctoral Thesis.

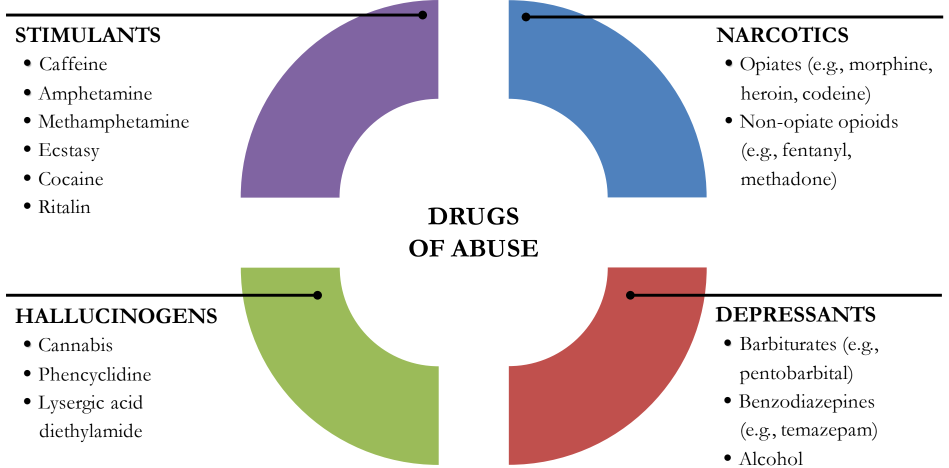



**Figure 2.** Types of commonly (ab)used DOAs.

## STIMULANTS

Stimulants are drugs that elevate one's mood because they increase the activity in the brain. In this sense, stimulants make everyone feel better, even if they are sad or depressed. They give people extra energy. Other claims are also made with respect to stimulants, such as they make you stronger, faster, have better sexual experiences and even smarter. For these reasons, stimulants are also referred to as *uppers* [8, 9]. The most commonly-used street drugs that fall into this category are amphetamines and cocaine.

Amphetamines refer to a group of low-molecular-weight basic drugs, the principal members of which include amphetamine and methamphetamine. In addition to these drugs, other substances also fall into this group, such as fenethylline, ephedrine, pseudoephedrine, methylphenidate, 3,4-methylenedioxymethamphetamine (commonly known as ecstasy) and methcathinone, as well as other several derivatives [10]. Broadly, amphetamines (brand names: Adderall®, Dexedrine, etc.) are commonly prescribed to treat attention deficit hyperactivity disorder, narcolepsy and, occasionally, depression. However, due to their stimulant activity, their abuse has risen worldwide. As shown in Figure 3, there were between 19 million and 36 million users of amphetamines around the world in 2014 [4]. Street names for amphetamines include speed, base, dexies, bennies, ice, crank, whizz, crystal meth, etc. Amphetamines are usually found in pills and often crushed for snorting.

**Figure 3.** Schematic representation of the global number of amphetamines users in 2014 [4].

Cocaine is a natural product extracted from the leaves of the coca plant (*Erythroxylum coca Lamarck* and *Erythroxylum novogranatense*). These tropical shrubs are cultivated widely on the Andean-Amazonian ridge in South America, particularly in Bolivia, Colombia and Peru. When the pure drug was first extracted from the leaves in the

19th Century, its harmful effects, mainly addiction, were not known. In the early 20th Century, it was legally sold in medicines and soft drinks. Nowadays, cocaine is one of the most notorious DOAs. According to the UNODC's 2016 World Drug Report, there were around 19 million users of cocaine in 2014 worldwide [4]. Its street names include coke, snow, stardust, nose candy, white, charlie, etc. Cocaine is commonly found in the form of a white powder that is often mixed with other chemicals, such as baking soda or baby powder, as fillers.

## NARCOTICS

Narcotics are drugs that dull the senses and induce sleep. In medical use, the term narcotic refers to opiates. Opiates are a group of natural, semi-synthetic and synthetic alkaloid compounds derived from the latex of the opium poppy plant (*Papaver somniferum*). Natural opiates include morphine, codeine and thebaine. Other substances, called opioids, also fall into this group and are man-made. These substances include pethidine, oxycodone, hydrocodone, fentanyl, buprenorphine and methadone. Both opiates and opioids are generally used to treat chronic or severe pain. However, the reality is that the abuse of these pain medications has become epidemic, as can be observed in Figure 4 [4]. This abuse does not only occur in the case of drug users, but may also start with patients seeking legitimate treatment for an actual condition. Heroin is a manufactured opioid and has no medicinal uses. It is mainly used for its ability to give the user a feeling of euphoria.

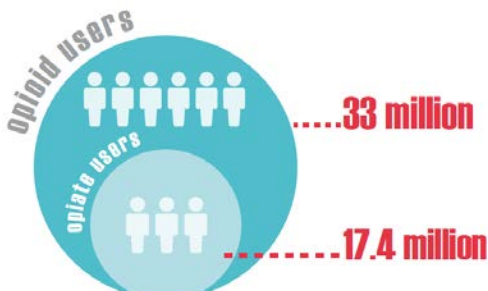



**Figure 4.** Schematic representation of the global number of opiate/opioid users in 2014 [4].

## HALLUCINOGENS

Hallucinogens are drugs that distort the mental and perceptual abilities of people, causing bizarre images and sensations. These sensations are so unusual that they are

sometimes referred to as *trips*. Users often see images, hear sounds and experience sensations that seem realistic but do not exist. For example, a person using a hallucinogen may feel as though bugs are crawling over their entire body when there are, in fact, no insects on them at all. Hallucinogens can be found naturally in plants, seeds, fungi and leaves, or they can be man-made [5, 8]. The most common hallucinogens include lysergic acid diethylamide, mescaline, psilocybin, phencyclidine and cannabis drugs (marijuana and hashish). Actually, cannabis drugs are the most widely consumed naturally occurring drugs throughout the world. About 183 million people are estimated to have used cannabis in 2014 (Figure 5). These drugs produce a feeling of relaxation and euphoria, but also anxiety and paranoia are possible, as well as short-term memory loss. The primary psychoactive component of cannabis drugs is $\Delta^9$-tetrahydrocannabinol. Apart from recreational use, $\Delta^9$-tetrahydrocannabinol also has therapeutic benefits as an analgesic, being prescribed (as Marinol) in some countries for the treatment of nausea in cancer chemotherapy and to people with chronic pain.

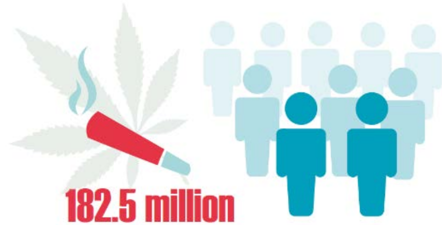



**Figure 5.** Schematic representation of the global number of cannabis users in 2014 [4].

## DEPRESSANTS

In contrast to the stimulants, depressants are drugs that produce a calming effect in the body and slow down the brain's normal functioning. In this respect, depressants decrease the level of awareness, lower the pulse and heart rate, and reduce the breathing rate. For these reasons, depressants are also referred to as *downers*. Taken in small doses, depressants cause relaxation and calmness, which can result in a reduction in anxiety and lower inhibitions, whereas, in large doses, they can have serious consequences, including complete loss of consciousness, loss of senses, slurred speech, respiratory depression and even death [5, 8]. The most commonly abused depressants are barbiturates and benzodiazepines.

Barbiturates are a group of chemical substances based on the compounds barbituric acid and thiobarbituric acid. Barbituric acid itself is not a central nervous system depressant, but barbital derived from barbituric acid has sedative-hypnotic properties. Within this context, over 2,500 derivatives have been produced over the years. Based on the duration of the sedative effect, barbiturates are classified as ultra short-, short-, intermediate- and long-acting. Ultra short-acting compounds, such as thiopental and methohexital, are used for anaesthesia, while long-acting compounds, such as phenobarbital and mephobarbital, are used as anticonvulsants. The short- and intermediate-acting compounds, such as amobarbital, butalbital, butabarbital, pento-barbital, secobarbital and talbutal, are used for treating insomnia and, occasionally, to perform preoperative sedations. Usually short- and intermediate-acting barbiturates are abused, while long-acting barbiturates are rarely misused [6, 9, 10].

Benzodiazepines are the most widely prescribed drugs worldwide. Benzodiazepines, like barbiturates, can be short- and long-acting. Short-acting benzodiazepines, such as flurazepam, temazepam, triazolam and estazolam, are generally prescribed to treat insomnia, while long-acting benzodiazepines, such as alprazolam, chlordiazepoxide, clorazepate, diazepam, halazepam, lorazepam, oxazepam, prazepam and quazepam, are used for treating both insomnia and anxiety disorders. In addition, benzodiazepines, such as clonazepam, diazepam and clorazepate are used as anticonvulsants. The most commonly abused benzodiazepines are alprazolam, diazepam, lorazepam, oxazepam and triazolam [9, 10].

## NEW PSHYCHOACTIVE SUBSTANCES

Over the last decade, and particularly in the last five years, an increasing number of new substances have been detected on European drug markets, with two new substances detected every week. Under European Union law, these drugs are known as NPS and they constitute a broad group. Currently, over 560 NPS are being monitored by the European Monitoring Centre for Drug and Drug Addiction with 100 new substances reported for the first time in 2015 [7]. NPS are types of synthetic drugs that mimic effects of the classic DOAs, such as marijuana, heroin, cocaine, amphetamines, benzodiazepines and lysergic acid diethylamide, but which are not scheduled under the international drug control conventions [4, 7]. Within this context, NPS production appears to take place primarily in legitimate chemical companies in China and India. The drugs are then imported into Europe, where they are sold directly on the black market or packaged and marketed as legal highs, research

chemicals and food supplements and openly sold (Figure 6). Synthetic cannabinoids and synthetic cathinones are the largest groups on the NPS scene [11].

**Figure 6.** From synthesis to consumer [7].

## 1.2. DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES

Today, drug testing in biological specimens is one the most common ways to prove and determine drug abuse. The most widely used biosamples in adults (aged 15 to 64 years) for this purpose include urine, blood, oral fluid (saliva), sweat and hair. All these matrices are equally important for drug testing as each has its own characteristics and detection time for DOAs [12-14]. A summary of these issues is provided in Table 1.

**Table 1.** Summary of detection times, advantages and disadvantages of different biological samples used for drug testing.

| *Matrix* | *Detection time* [a] | *Advantages* | *Disadvantages* |
|---|---|---|---|
| Urine | Few minutes-hours-days-weeks (depending on drug of abuse, see Table 3) | • Generally, available in large quantities<br>• DOAs and/or their metabolites are highly concentrated<br>• Well-researched testing techniques<br>• Availability of on-site testing devices<br>• Relatively inexpensive | • Easy to adulterate, dilute or substitute<br>• May require observed collection<br>• Potential for shy bladder syndrome, therefore cannot produce a specimen<br>• Drug levels influenced by fluid intake<br>• Unstable if not properly stored (refrigeration or freezing storage) |
| Blood | Few minutes-hours (depending on drug of abuse) | • Difficult to adulterate<br>• Drug levels in blood show good correlation to drug effects on an individual's functioning and behaviour | • Invasive collection (venipuncture)<br>• Requires medical staff to collect specimen<br>• Risk of infection<br>• May not be an option for individuals with poor venous access<br>• Limited sample volume can be obtained<br>• No on-site testing devices<br>• Unstable if not properly stored (refrigeration or freezing storage) |
| Sweat (patch) | Few hours-days [b] | • Non-invasive collection<br>• Difficult to adulterate<br>• Cumulative measure<br>• Relatively tamper-proof collection method | • Contamination during patch handling<br>• Risk of accidental or deliberate removal of the patch<br>• High variation in sweat production<br>• No on-site testing devices |

**Table 1.** (Continued).

| *Matrix* | *Detection time* [a] | *Advantages* | *Disadvantages* |
|---|---|---|---|
| Saliva | Few minutes-hours-days (depending on drug of abuse) | • Observed, non-invasive collection<br>• Easy to collect<br>• Reduced risk of adulteration<br>• Availability of on-site testing devices<br>• Generally, drug levels in saliva correlate well with levels found in the blood | • Limited volume<br>• Risk for passive contamination<br>• Salivation reduced by stimulant use<br>• Drug adsorption to collection device |
| Hair | Few days-weeks-months-years [c] | • Observed, non-invasive collection<br>• Easy to collect, handle, store, and transport<br>• Difficult to adulterate or substitute<br>• The second specimen can be obtained from original source | • Cannot detect recent drug usage<br>• Racial/ethnic and hair colour bias<br>• Possible environmental contamination<br>• Can be removed by shaving<br>• Testing may not detect single drug usage<br>• No on-site testing devices |

[a] Besides the matrix, the detection time depends on the dose, route of administration, short-term versus long-term use, the physicochemical properties of the substances, etc. [12, 13]. [b] As long as the patch is worn, usually seven days [14]. [c] Hair has been assumed that grows at a rate of around 1 cm per month, therefore dependent on hair length at the time of the sampling [15].

In view of the above, individually or collectively, all of the biological samples mentioned can be used to identify use and/or exposure to DOAs effectively in scenarios such as:

- Drug testing by employers to detect the use of DOAs.
- Screening of athletes and sportsmen to detect any illicit performance-enhancing DOAs.
- Testing by police officers, e.g., testing for illicit substances in the case of people who seem to be driving under the influence of DOAs.
- Insurance companies carrying out drug testing before letting an individual purchase a plan.
- Testing as a result of court orders, usually in cases of child custody or after allegations of date rape drug abuse.

- Testing by health professionals to ascertain causes of a disease the individual is suffering from.
- Drug testing to monitor a person who is abstinent and has joined a support program.

The choice of the matrix will be influenced by the cost, ease of sample collection, risk of adulteration, test type (instant or laboratory based), scope of DOAs being tested, time frame (acute or chronic use), time since last use and indications for testing [16].

The present Doctoral Thesis focuses on the development of analytical drug testing procedures in hair and urine samples. Therefore, an overview is provided of issues related to the collection, analysis and interpretation of the results found when different DOAs are determined in these matrices.

### 1.2.1. HAIR DRUG TESTING

Hair analysis has received increased attention in recent years and, currently, it has become the third most commonly used biological matrix for drug testing, after blood and urine, shown by the large number of application developed [15]. For instance, Figure 7 shows the evolution in the number of cases analysed in a Spanish forensic laboratory, from 2004 to 2013 [17]. A total of 6,000 cases were analysed during these 10 years. The increasing interest is certainly based on the unique advantages of hair drug testing that are outlined in Table 1. Hair samples are routinely collected and analysed to address a wide range of challenges, such as drug abuse history, workplace drug testing, drug-related deaths, child protection, therapeutic drug monitoring, doping control and drug-facilitated assault investigations. Within this context, a large variety of literature related to different applications of hair testing for DOAs has been published to date.

Recently the journal *Analytica Chimica Acta* reported our review paper related to the state of the art and the latest trends used in the literature from 2005 to 2015 in the analysis of DOAs in hair, with a special focus on separation analytical techniques and their hyphenation with mass spectrometry detection (MS). The most recently introduced sample preparation and preconcentration techniques are also addressed in this publication. In addition, the main strengths and weaknesses of all of these approaches are critically discussed by means of relevant applications, as well as numerous considerations concerning the interpretation of hair drug testing results are included.

A copy of this manuscript is attached in Section 1.2.1.1. of the present Doctoral Thesis.

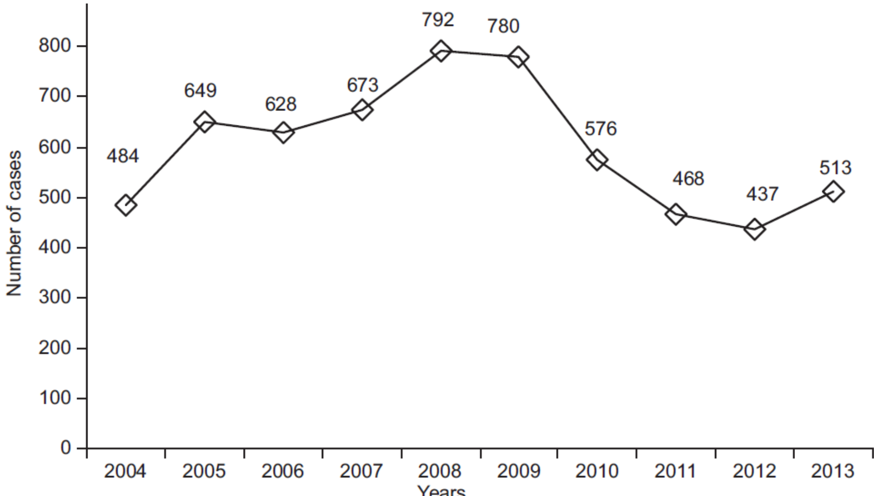



**Figure 7**. Evolution in hair analysis-related cases in a Spanish forensic laboratory over 10 years [17].

However, over the last year (2016-June 2017), several other research articles dealing with hair drug testing have appeared in the scientific field {Table 2 [18-34]} which have not been included in our review and, therefore, will be summarized below.

As can be seen from the table, several hair analysis methods using gas chromatography (GC)-MS have been published with limits of detection (LODs) in the low ng/mg concentration range [27-32]. Liquid chromatography-tandem MS (LC-MS/MS) is another analytical technique extensively used, offering high signal-to-noise ratio and selectivity [18-26]. Very recently, ambient MS ionization techniques, such as direct analysis in real time (DART), have also been introduced allowing direct ionization at atmospheric pressure to analyse hair samples in their native state [33, 34]. The main advantage of the latter is the complete lack of sample preparation procedures, enabling very short analysis times.

An important issue in hair analysis is the decontamination technique used prior to the extraction of drugs from the hair fibres. In this regard, the great majority of the publications included in Table 2 used a decontamination technique to remove substances loosely attached to the hair surface. However, there is no methodology

**Table 2.** Methodologies reported in the literature over the last year (2016-June 2017) for the analysis of DOAs in hair.

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mg)* | *Ref.* |
| --- | --- | --- | --- | --- |
| KET, NKET | *LC-ESI-MS/MS (IT)* Stationary phase: Accuore phenyl-hexyl, 150 × 2.1 mm, 2.6 µm at 30ºC with a matching guard column, 10 × 2.1 mm; Mobile phase: Gradient; (A) 10 mM ammonium acetate, (B) ACN; Flow rate: 0.2 mL/min | 30 mg pulverized hair; Washing with DCM, acetone; Extraction with phosphate buffer at pH 6.2 (10 min, 50ºC); SPE with Strata-X-C (mixed-mode); Evaporation/ reconstitution | 0.008 (KET); 0.015 (NKET) | [18] |
| Psychotropic phenyl alkyl amines and KET | *LC-ESI-MS/MS (QTrap)* Stationary phase: Scherzo SS-$C_{18}$, 150 × 3 mm, 3 µm at 40ºC; Mobile phase: Gradient; (A) 35 mM AA/ 90% ACN in water, (B) 10 mM ammonium acetate/ 90% ACN in water; Flow rate: 0.5 mL/min | 5-10 mg hair; Washing with MeOH, water, MeOH; Extraction with 1% HCl in MeOH (20 h, 38ºC); Evaporation/reconstitution | 0.02-0.10 | [19] |
| Fifty psychoactive DOAs and metabolites | *LC-ESI-MS/MS (QTrap)* Stationary phase: Kinetex® Biphenyl, 50 × 2.1 mm, 35 µm with an equivalent guard column 2 × 2 mm; Mobile phase: Gradient; (A) 0.1% FA in water with 1 mM ammonium formate, (B) ACN/MeOH (70:30, v/v) with 0.1% FA in water/1 mM ammonium formate; Flow rate: 0.25 mL/min | 50 mg pulverized hair; Washing with acetone, hexane; Incubation in MeOH (overnight, 45ºC); QuEChERS; Evaporation/ reconstitution | 0.002-0.020 | [20] |
| AB-CHMINACA and its metabolites | *LC-ESI-MS/MS (QTrap)* Stationary phase: Zorbax Eclipse Plus $C_{18}$ RRHD, 100 × 2.1 mm, 1.8 µm with an equivalent guard column at 40ºC; Mobile phase: Gradient; (A) 2 mM ammonium formate in 0.2% FA in water (B) 2 mM ammonium formate in 0.2% FA in MeOH; Flow rate: 0.3 mL/min | 10 mg hair; Washed with MeOH, water, MeOH; Incubation in MeOH (20 h, 38ºC); Evaporation/reconstitution | 0.0005-0.0100 | [21] |

**Table 2.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mg)* | *Ref.* |
|---|---|---|---|---|
| 4-MEC, MDPV | *LC-ESI-MS/MS (QqQ)* Stationary phase: Hypersil GOLD perfluorophenyl, 100 × 2.1 mm, 1.9 µm at 30ºC; Mobile phase: Gradient; (A) ACN, (B) Formate buffer; Flow rate: 0.3 mL/min | 20 mg pulverized hair; Washing with DCM, warm water; Incubation in phosphate buffer pH 5 (10 min, 95ºC); LLE with hexane/EtAc (1:1, v/v); Evaporation/reconstitution | 0.0005 | [22] |
| Classic ATS, synthetic cathinones and synthetic piperazines | *LC-ESI-MS/MS (QqQ)* Stationary phase: Atlantis® T3, 50 × 2.1 mm, 3 µm at 35ºC; Mobile phase: Gradient; (A) 2 mM ammonium formate in 0.1% FA pH 3, (B) ACN; Flow rate: 0.3 mL/min | 30 mg pulverized hair; Washing with DCM (three times); Incubation in 0.1% HCl in MeOH (1 h, 60ºC); SPE with Strata-X-C (mixed-mode); Evaporation/ reconstitution | 0.0002-0.0050 | [23] |
| AP, MP, MDMA, MDEA, MOR, COD, 6-AM, MTD, EDDP, COC, BZE, EME | *LC-ESI-MS/MS (QqQ)* Stationary phase: EZ:faast $C_{18}$, 250 × 2 mm, 4 µm; Mobile phase: Gradient; (A) 5 mM ammonium formate with 0.01 % FA in water (B) 0.01% FA in ACN; Flow rate: 0.3 mL/min | 20 mg pulverized hair; Washing with water, acetone; Incubation in MeOH/ACN/ 2 mM ammonium formate in water pH 5.3 (25:25:50, v/v/v) (18 h); Injection into the system | LOQ: 0.025 | [24] |
| Thirty-one stimulant, psychedelic and dissociative designer drugs | *UHPLC-ESI-MS/MS (QTrap)* Stationary phase: Zorbax Eclipse Plus $C_{18}$ RRHD, 100 × 2.1 mm, 1.8 µm with a $C_{18}$ pre-column at 45ºC; Mobile phase: Gradient; (A) Water/ 5 mM FA, (B) ACN/MeOH (80:20, v/v) with 5 mM FA; Flow rate: 0.5 mL/min | 25 mg hair; Washing with DCM, MeOH; Incubation in MeOH (15 h, 55ºC); Injection into the system | 0.0009-0.0170 | [25] |
| KET, NKET | *UHPLC-ESI-HRMS (Orbitrap)* Stationary phase: Atlantis® T3, 150 × 2.1 mm, 3 µm; Mobile phase: Gradient; (A) 0.1% FA in water, (B) 0.1% FA in ACN; Flow rate: 0.3 mL/min | 10 mg pulverized hair; Washing with MeOH, isopropanol, phosphate buffer; LLE with water/ MeOH/1 M TFA (80:10:10, v/v/v); Injection into the system | 0.003 | [26] |

**Table 2.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mg)* | *Ref.* |
|---|---|---|---|---|
| MOR, COD, 6-AM, COC, EME, BZE, AP, MP, MDMA, MDEA | *GC-EI-MS* Column: HP-5MS, 30 m × 0.25 mm, 0.25 µm f.t.; Carrier gas: helium | 30 mg hair; Washing with water, acetone, DCM; Incubation in MeOH (2 h, 50ºC); Derivatization with BSTFA + 1% TMCS | 0.05-0.46 | [27] |
| GHB | *GC-EI-MS* Column: HP-5MS, 30 m × 0.25 mm, 0.25 µm f.t.; Carrier gas: helium | 25 mg hair; Washing with DCM; Digestion with 1 M NaOH (20 min, 90ºC); LLE with EtAc; Derivatization with BSTFA + 1% TMCS in EtAc | 0.05 | [28] |
| Pheno-barbital, secobarbital, pentobarbital | *GC-EI-MS* Column: HP-5MS, 30 m × 0.25 mm, 0.25 µm f.t.; Carrier gas: helium | 50 mg hair; Washing with DCM, water, mild detergent; Hydrolysis with 1 M NaOH (15 min, 70ºC); HF-LPME; Deriva-tization with TMAH + EtAc | 0.1 | [29] |
| COC and its derivatives | *GC-EI-MS* Column: HP-5MS, 30 m × 0.25 mm, 0.25 µm f.t.; Carrier gas: helium | 50 mg hair; Washing with detergent, water, DCM; Incubation in MeOH (18 h, 50ºC); Derivatization with ACN + Pyr + butyl chloroformate; HF-LPME; Evaporation/ reconstitution | 0.03-0.40 | [30] |
| THC, heroin, COC, ATS and derivatives | *GC-EI-MS* Column: VF-5MS, 25 × 20 mm, 0.33 µm f.t.; Carrier gas: helium | Washing with detergent, water, MeOH, acetone, diethyl ether; *For THC:* 50 mg pulverized hair; Incubation in 1 M NaOH (30 min, 80ºC); LLE; Derivatization with TMSH + MeOH; *For other drugs:* 35 mg pulverized hair; Incubation in 0.1 M phosphate buffer pH 6 (4 h); SPE with Chromabond Drug; Derivatization with TFA + HFIP | 0.0019-0.0059 | [31] |

**Table 2.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mg)* | *Ref.* |
|---|---|---|---|---|
| PMA, KET, NKET | *GC-EI-MS*<br>Column: HP-5MS, 30 m × 0.25 mm, 0.25 μm f.t.; Carrier gas: helium | 10 mg hair;<br>Washing with MeOH, water, MeOH; Incubation in 1% HCl in MeOH (20 h); Derivatization with EtAc + TFA | 0.25-0.50 | [32] |
| Multiple common DOAs | *DART-HRMS (Orbitrap)* | 10 mg hair<br>(150-200 hairs) | – | [33] |
| THC | *DART-MS/MS (QqQ)*<br>*DART-HRMS (QTOF, Orbitrap, Q Orbitrap)*<br><br>A comparison study of the different mass analysers | 10 mg hair<br>(150-200 hairs) | – | [34] |

See Appendix I for the key to abbreviations.

that has gained universal acceptance. There are different washing strategies reported in the literature and all of them usually consist of using a solvent or a mixture of solvents, and the general purpose is that these ones should remove external impurities as fully as possible, but without extracting the drugs from the hair matrix [35].

With regard to the release of drugs bound within the hair matrix, no important achievements were reported, with this commonly being performed either by alkaline digestion [28, 29, 31], solvent extraction [19-21, 23-25, 27, 30, 32] or incubation with phosphate buffer systems [18, 22, 31]. After the extraction procedures, clean-up of the hair extracts and/or preconcentration of the target compounds is usually performed either by liquid-liquid extraction (LLE) [22, 26, 28, 31] or SPE [18, 23, 31]. More recently, QuEChERS has also proven to be suitable for this purpose, simplifying and reducing the time of extraction and clean-up compared to LLE and SPE procedures [20]. In addition, great attention has been paid in recent years to modern microextraction techniques, mainly due to the low consumption of organic solvents (low μL range). In this respect, hollow-fibre liquid-phase microextraction (HF-LPME) was used in conjunction with GC separation for hair drug testing, demonstrating to be suitable for the intended task [29, 30].

The following section includes our review article titled "Recent trends in analytical methods and separation techniques for drugs of abuse in hair" published in the journal *Analytica Chimica Acta*.

#### *1.2.1.1. Recent trends in analytical methods and separation techniques for drugs of abuse in hair*

UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES
Tatiana Baciu

# RECENT TRENDS IN ANALYTICAL METHODS AND SEPARATION TECNIQUES FOR DRUGS OF ABUSE IN HAIR

**Tatiana Baciu, Francesc Borrull, Carme Aguilar* and Marta Calull**

Department of Analytical Chemistry and Organic Chemistry, Rovira i Virgili University, Campus Sescelades, Marcel·lí Domingo, 1, 43007 Tarragona, Spain

**Abstract**

Hair analysis of drugs of abuse has been a subject of growing interest from a clinical, social and forensic perspective for years because of the broad time detection window after intake in comparison to urine and blood analysis. Over the last few years, hair analysis has gained increasing attention and recognition for the retrospective investigation of drug abuse in a wide variety of contexts, shown by the large number of applications developed. This review aims to provide an overview of the state of the art and the latest trends used in the literature from 2005 to the present in the analysis of drugs of abuse in hair, with a special focus on separation analytical techniques and their hyphenation with mass spectrometry detection. The most recently introduced sample preparation techniques are also addressed in this paper. The main strengths and weaknesses of all of these approaches are critically discussed by means of relevant applications.

***Keywords**: hair analysis/drug of abuse/gas chromatography/liquid chromatography/capillary electrophoresis/sample preparation*

## 1. Introduction

Drugs of abuse are a major concern that is increasingly affecting every sector of society. Consequently, there is a need for the continuous development of methods for the efficient determination of drugs of abuse and their metabolites in biological samples. The benefits of hair analysis are numerous [1–12]. The ability to detect past drug consumption is a unique feature of this matrix, as it provides researchers with a longer detection window (months to years). Assuming hair grows approximately 1 cm per month, segmental analysis of hair strands allows the determination of the historic pattern of drug use [4, 13–18]. Additional advantages of testing hair include a non-invasive means of easily supervised sample collection, reduced risk of sample adulteration, easy sample storage and transportation, and reduced risk of exposure to biohazards. As such, hair analysis of illicit drugs and pharmaceuticals is currently employed to address a wide range of challenges including drug abuse history, workplace testing, post-mortem toxicology, therapeutic drug monitoring and drug facilitated assault (DFA) investigations [1, 3, 4, 7, 10, 11, 19–21].

The mechanism of incorporation of xenobiotics into hair has not yet been clearly defined, but there are several potential mechanisms, as shown schematically in Figure 1, which are generally accepted among the scientific community: passive diffusion from blood capillaries during hair formation, deposition by diffusion from sebum or sweat secretions into the hair follicle after formation, and from the external environment [3, 5–26]. According to the passive diffusion model, excretion of drugs in hair should be delayed a few days because new hair takes some time to emerge from the skin surface and be available for sampling [6, 14, 21, 27–29]. The chemical properties of the incorporated drugs, as well as the physical/physiological characteristics of the individual, strongly influence which mechanism will dominate. Three key factors influence drug incorporation. These are the melanin content of hair and the lipophilicity and basicity of the substance itself: pigmented hair will contain higher drug amounts and, lipophilic and basic drugs will be more concentrated [3, 5, 9, 11, 22, 30, 31]. Externally, drugs can be deposited on hair from the environment via smoke, pollution or physical contact, chemicals, etc. (Figure 1), leading to false-positive results [9, 26, 29, 32, 33]. With this in mind, the Society of Hair Testing

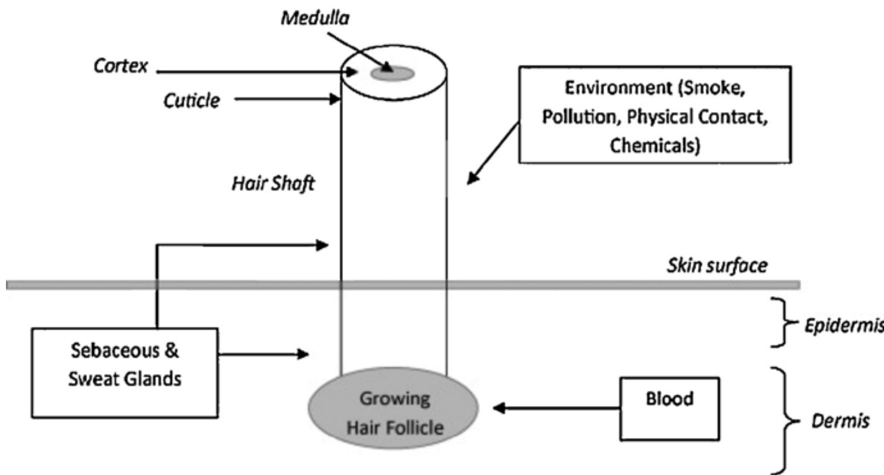



**Figure 1.** Schematic of drug incorporation into/onto the hair shaft. Reproduced from [215] with the permission of Elsevier, copyright 2010.

(SoHT) and Substance Abuse and Mental Health Services Administration (SAMHSA) produced a set of recommendations (wash-out analysis, metabolite identification, cut-off values) that were shown to be helpful for an appropriate interpretation of the results [19, 20]. Later in this paper, there is a discussion of the fundamental factors to take into consideration when interpreting analytical results (external contamination, cosmetic treatments, hair colour, differences in hair growth rate).

In the case of abusers, most drugs are expected to be found in hair in the ng/mg range; cannabinoids are usually found at lower concentrations (pg/mg) and 11-nor-$\Delta^9$-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) is even less concentrated. In recent years, it has been shown that a single exposure to a drug is detectable in hair in the case of most drugs, with concentrations in the low pg/mg range being expected, particularly, in DFA cases because drugs are frequently administrated surreptitiously in beverages [2, 34, 35].

Drug-associated crimes (sexual assault, robbery) are now frequently reported. Drugs used to facilitate sexual assaults are pharmaceuticals such as benzodiazepines (BZDs), hypnotics, sedatives and anaesthetics, or illegal drugs, and they are secretly administrated (usually through alcoholic drinks) to the victim prior to the assault. After a single dose, they can be difficult to detect, because of their low dosage, chemical instability, amnestic properties and

short half-lives in the human body. Hair is suggested as a valuable specimen in situations where, as a result of a delay in reporting the crime, natural processes have eliminated the drug from conventional biological specimens. Moreover, embarrassment associated with urine collection, particularly after sexual assault, can be mitigated by hair analysis [36, 37].

3,4− Methylenedioxymethamphetamine (MDMA), also known as ecstasy, has been widely used for several decades and abuse of this designer drug is increasing rapidly among young people. MDMA tablets seized by law-enforcement officials can contain a variety of components as a drug cocktail. Identified components of tablets included 3,4-methylenedioxyamphetamine (MDA), methamphetamine (MP), amphetamine (AP), ketamine (KET), cocaine (COC), caffeine, phencyclidine (PCP) and ephedrine. Consumption of these tablets has the potential for multidrug abuse. Most subjects who tested positive for other components reported being unaware they had ingested any drug other than MDMA. Hair analysis can reveal exposure to unexpected drugs [2]. Aside from the classic drugs of abuse, new psychoactive substances play a big role. The cathinones including beta-keto amphetamines (methylone, butylone, ethylone), methcathinone and mephedrone as well as the piperizines [such as benzylpiperazine (BZP), 1-(3-chloro-phenyl) piperazine (mCPP) and 1-(3-trifluoromethyl-phenyl) piperazine (TFMPP)] are often sold via internet under the guise of bath salts or plant food [38, 39].

In view of this, the determination of various classes of abused drugs in hair is very important in many fields of analytical toxicology. The detection method must provide accurate and reliable results from the test sample. In this regard, many procedures have been published in the scientific literature. On this matter, several comprehensive reviews focusing on drug analysis have been reported in the scientific literature, in which sections are organized by different classes of drugs [5, 7, 8, 18, 30, 34, 40–44], matrices (including hair) [4, 9–12, 22, 23, 45, 46] and analytical methods employed [6, 29, 36, 47–58].

Typically, the analytical methodologies employed for the determination of drugs in hair samples consist of different steps: (1) sampling, (2) segmentation when a segmental analysis of hair sample is carried out, (3) decontamination by washing the hair samples to eliminate any possible external contamination, (4) pulverization in a ball-mill or cutting into small pieces, (5) extraction of the drugs and their

metabolites from the hair matrix, (6) clean-up of hair extracts and preconcentration of the target compounds, (7) determination of the drugs by means of different separation techniques coupled to different detectors and, finally, (8) interpretation of the analytical findings [5–9, 52]. The quality of the obtained results depends on the correct performance of each one of these steps [8, 23].

In order to conduct article research as extensively as possible, literature searches were performed using the following electronic databases: PubMed, ScienceDirect, Scopus and SciFinder. The key terms used were: biological samples, hair structure and formation, drugs of abuse, hair drug testing, toxicological analysis, forensic and clinical analysis, illicit drugs analysis, hair sample preparation, hair collection, methodology for hair drug testing, segmental hair analysis, chromato-graphic procedures for hair analysis, drug monitoring, pitfalls in hair analysis, bias in hair drug testing, melanin bias in hair drug testing, and new designer drugs, among others. The criterion for selecting was limited to publications focused on different aspects related to human hair analysis for drugs of abuse in the period from 2005 to the present. A few studies published before 2005 have also been selected and discussed, as they were considered relevant for a better understanding and interpretation of hair analysis results.

## 2. Hair sampling and storage

The site of choice for hair collection is head hair for most investigators, as body hair growth is more variable and substantially slower than head hair, leading to difficulty in interpreting time of use for suspected users. Besides, using non-head hair presents additional challenges related to collection and invasiveness [21, 59–62]. For forensic purposes, the estimation of the time of drug intake is of great importance, as it enables us to ascertain whether or not the crime has been committed under the influence of drugs. It is usually stated that head hair grows at a rate of approximately 1 cm per month [13–15] and therefore, it is possible to link the presence of a drug in a segment of hair to a certain period in the past. However, due to variables such as seasonal effects, age, pregnancy and hormones, variability in hair growth rates has been observed ranging from 0.7 to 1.3 cm per month [6, 13, 14, 22, 63]. Additionally, a recent report by LeBeau *et al.* [21] suggests that on average 0.8 cm hair is left on the scalp after sampling with scissors. Taking into account this amount of hair left on the scalp, as well as an average

linear growth rate of 1 cm per month, and the assumption that it takes two weeks for newly formed hair in the follicle to reach the scalp [64], the authors recommend that hair collection be delayed until eight weeks after suspected ingestion to ensure that the sample fully represents the exposure period.

In DFA cases, the hair sample should be collected approximately one month after the event. As it is important to detect a single exposure to the drug, the distal segments (corresponding to an older period in the past) should produce a negative result, whereas the administered drug would be found approximately 1 cm from the hair root [14].

When a hair sample is received in the laboratory, it often appears with the cut ends of the hair bundle misaligned. It is common practice to line up and assign a zero value to the cut end. However, this assumption is an underestimation of the actual range of time that the cut end of the hair sample represents, which is important to take into consideration in interpreting results of segmental hair analysis [21]. Consequently, it is not advisable to collect more than 60 hair fibres per strand. Otherwise, there is a risk of collecting in an oblique way, i.e. with hair grown in different periods [21].

With respect to sampling, in general, the most recommended sampling site is the back of the head, in the vertex posterior, where hair has less variability in growth and is less influenced by age and sex. Figure 2 shows a typical protocol for hair sample collection. The hair sample should be tied with thread (A), before being cut as close as possible to the scalp, making sure the scissors are levelled with the scalp (B). The sample size taken is dependent on the purpose of the analysis. Usually between 20 and 200 mg of hair (often described as a lock of hair or a pencil thickness of hair) is sufficient for screening and confirmation of drugs. Once collected, the hair locks must be rolled into separate aluminium foils placed with the root end exposed beyond the end of the foil as shown below (C). This procedure attempts to avoid misalignment of a hair sample and allow easy identification of the root end, which is important for segmental analysis. Each aluminium foil is folded once or twice (D) and subsequently stored in paper envelopes at room temperature in a dry, dark place (E). Lastly, information about the case history and the hair sample characteristics should be obtained (F). Pragst and Balikova [6] recommend avoiding direct storage in plastic bags or plastic tubes owing to the risk of contamination by softeners, since

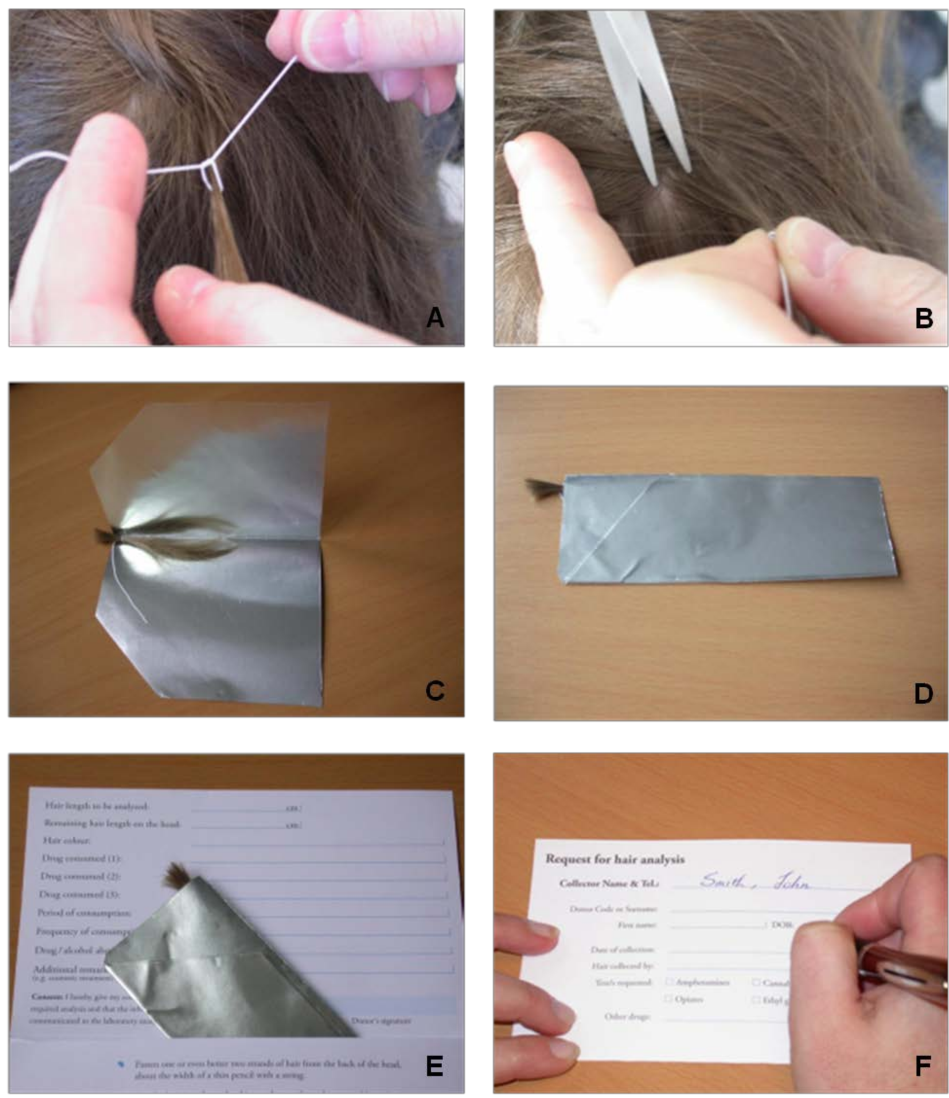



**Figure 2.** Procedure followed for hair sampling. The different steps are discussed in the text.

plastic can potentially extract lipophilic substances from the hair, and because of the risk of a plastic tube being broken during shipping or storage manoeuvres. When sectional analysis is performed, hair is cut into segments of about 1, 2 or 3 cm, which correspond to about one, two or three months' growth [7, 8, 19, 23, 40, 65].

## 3. Hair sample preparation

Sample preparation is a fundamental part of any analytical method and has a significant influence on most of the subsequent analytical steps and on data quality. Sample preparation for hair usually requires a long procedure due to the complexity of this kind of matrix. To prepare a hair sample for analysis, a wash procedure has to be applied to eliminate any possible external contamination and then an extraction procedure of the drugs and their metabolites is necessary. In this latter step, the drugs are released from the hair matrix and then an additional clean-up step is required in order to eliminate the possible interfering substances. Furthermore, a preconcentration step has to be included in some cases to preconcentrate the target compounds. The quality of the results depends on the correct performance of each one of these steps, which are summarized in Tables 1–3.

### *3.1. Decontamination*

In order to improve analytical performance, residues from hair care products, sweat, sebum or even dust should be removed by washing the hair samples before analysis. Moreover, a wash procedure helps to avoid false-positive results by removing possible passive external environmental contamination of drugs [1, 11, 24, 32, 33, 40, 66]. There are different washing strategies reported in the literature, as can be seen in Tables 1−3. All of them usually include organic solvents, aqueous buffers, water, soaps or a combination. Decontamination strategies can take from minutes to hours to perform (typically between 2 and 10 minutes each washing) and the number of clean-up steps depends on the degree of suspected hair contamination [32]. The general purpose is that these procedures should remove external impurities as fully as possible, but without extracting the drugs from the hair matrix. There is no consensus on which procedure performs best and so each laboratory must optimize its own procedure. Upon being washed and dried, hair samples should be pulverized in a ball-mill or cut into small pieces prior to the drug solubilisation. The choice between the hair being powdered in a ball-mill or finely cut with scissors depends on the quantities of hair sample available, since, when hair sample is limited, usually the first option is preferred [19].

*3.2. Extraction/digestion*

Freeing the analytes bound within the hair matrix is commonly achieved either by acidic or alkaline digestion, solvent extraction, enzymatic hydrolysis, or incubation with various buffer systems, as shown in Tables 1–3. These approaches generally involve elevated temperatures and long incubation periods, typically between 16 and 20 hours. The choice depends on the chemical properties of the analytes. A pitfall in hair analysis is the inappropriate choice of the method at this stage, which could result in the degradation of the analytes of interest. For example, heroin, COC and BZDs are hydrolysed in alkaline conditions, while 6-acetylmorphine (6-AM), a heroin metabolite, may originate morphine (MOR) in an acidic environment [45, 67]. Recently, attention has focused on assisted extraction techniques, such as ultrasonic-assisted extraction (UAE) [68–72], microwave-assisted extraction (MAE) [73–76], supercritical-fluid extraction (SFE) [77] and pressurized-liquid extraction (PLE) [78], to accelerate and improve efficiency compared with the conventional methods mentioned above. Other novel approaches that have been investigated for the extraction of illicit drugs from hair to reduce instrumentation costs have included matrix solid-phase dispersion (MSPD) [72, 79] and micropulverized extraction [80–84].

*3.3. Clean-up and preconcentration*

Matrix constituents can be co-extracted with the analytes of interest and therefore interfere with the qualitative and quantitative analysis of studied substances. In this regard, after extraction procedures, sample pretreatment stages are usually carried out before instrumental analysis in order to clean up the hair extracts, as well as to preconcentrate the target compounds. In general, the most commonly applied sample preparation techniques for this purpose include traditional liquid–liquid extraction (LLE) and conventional solid-phase extraction (SPE), as shown in Tables 1–3. In recent years, highly clean extracts have become more important in attempts to prevent ion enhancement/ suppression when SPE extracts are analysed by different separation techniques coupled to tandem mass spectrometric detection using electrospray ionization (ESI). In this regard, new sorbents based on increased selectivity, such as molecularly imprinted polymers (MIPs) have been used for SPE to

remove matrix interferences in the determination of several drugs of abuse [85–87]. Miniaturized techniques are the current trend in the clean-up step, as they are considered green pretreatment techniques since they involve the reduction of organic [41], hollow-fibre liquid-phase microextraction (HF-LPME) [95] and microextraction by packed sorbent (MEPS) [84].

## 4. Hair analysis techniques

Different analytical methods mainly based on liquid chromatography (LC) or gas chromatography (GC) have been developed for the determination of drugs of abuse and their metabolites in hair samples. Electrophoretic/electrokinetic analytical strategies have also been applied successfully to drug testing in hair. In Tables 1–3, the most relevant papers regarding the determination of drugs of abuse in hair samples during the past few years are summarized and, in the following sections, a detailed discussion is given.

### *4.1. Liquid chromatography*

The polar character of most abused drugs makes them more suitable for analysis by LC than by GC since, in this case, a derivatization step prior to the analysis is not required. In this solvent volumes with the subsequent production of less toxic residues. Some of the most notable recent methodologies include headspace solid-phase microextraction (HS-SPME) [88–94], HS solid-phase dynamic extraction (HS-SPDE) regard, LC is an analytical technique widely used for toxicological analysis of hair samples as shown in Table 1. Reverse-phase high performance LC (RP-HPLC) is the most usual mode for separating and analysing drugs of abuse in hair. With respect to the detection system, mass spectrometry (MS) detection has been the most widely employed in recent years, although other detectors are still in use, as is the case of diode array detection (DAD) that has been employed for screening and quantification of some drugs of abuse, particularly in laboratories with no limited access to MS [73, 74]. However, due to the non-specific nature of DAD detection as well as the low concentrations of drugs found in hair, in the low pg/mg range, the use of highly sensitive and specific detection systems is required [96]. As can be seen from Table 1, LC coupled to tandem mass spectrometry (LC–MS/MS) using ESI or atmospheric pressure chemical ionization (APCI) [97, 98] is nowadays considered the method of choice for the analysis of drugs of

**Table 1.** Liquid chromatography-based methods for the determination of drugs of abuse in hair samples.

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| COC, BZE, COE, MOR, 6-AM, COD | HPLC-DAD | Washing with 0.1% Tween 80, water; MAE with MeOH (9 min, 60ºC) | LOQ: 200 | 50 | [73] |
| Nordoxepin, nortriptyline, imipramine, amitriptyline, doxepin, desipramine, clomipramine, norclomipramine | HPLC-DAD | Washing with 0.1% SDS, water, acetone, water; MAE with 1 M NaOH and hexane/isoamyl alcohol (40 min, 60ºC) | 300-1,200 | 45 | [74] |
| COC, BZE, COE, NCOC | LC-APCI-MS/MS (QqQ) | Washing with DCM (1.5 mL); Extraction with 25 mM phosphate buffer pH 2.7 (3 h, 75ºC); SPE (mixed-mode cation exchange, Cerex Clin II) | 25 | 10 | [97] |
| MOR, COD, 6-AM, AP, MP, BZE, COE, COC, MDA, MDMA, MDEA | LC-APCI-MS/MS (IT) | Washing with hexane, acetone; Extraction with MeOH (1 h, 50ºC) | LOD/ LOQ 50-200 | 20 | [98] |
| COC, BZE, COE, EME | HILIC-ESI-MS/MS (QqQ) | Washing with DCM, 0.01 M phosphate buffer, isopropanol, DCM; Digestion with 0.1 M HCl (overnight, 50ºC); SPE (mixed-mode cation exchange, Oasis MCX) | 0.5-1 | 10 | [120] |
| MOR, MP, 6-AM, AP, COD, THC, MDA, COC, MDMA, MTD, MDEA, BZE, buprenorphine | UHPLC-ESI-MS/MS (QqQ) | Washing with DCM (2 mL, 3 min, twice); Extraction with MeOH (15 h, 55ºC) | 6-27 | 50 | [121] |
| APs, BZDs, opiates, hallucinogens, antihistamines, antidepressants, antipsychotics, barbiturates | UPLC-ESI-MS/MS (QqQ) | Washing with isopropanol, water; Extraction with MeOH/ACN/ ammonium formate pH 5.3 (overnight, 37ºC); Filtration through a PTFE filter | LOQ: 2-5,000 | 10 | [122] |

**Table 1.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| 23 synthetic cannabinoids | UHPLC-ESI-MS/MS (QqQ) | Washing with DCM (2 mL, 3 min, twice); Incubation with 1 N NaOH (10 min, 95ºC); LLE with hexane/EtAc (90:10, v/v) | 1.3-80 | 10 | [123] |
| JWH-018, JWH-073, JWH-200, JWH-250, HU-210, THC, CBD, CBN | UHPLC-ESI-MS/MS (QqQ) | Washing with DCM (2 mL, 3 min, twice); Incubation in 1 N NaOH (10 min, 95ºC); LLE with hexane/ EtAc (90:10, v/v) | 0.02-5.4 | 50 | [124] |
| AP, MP, MDA, MDMA | UHPLC-ESI-MS/MS (QqQ) | Washing with isopropanol, 0.01 M phosphate buffer, isopropanol; Digestion with 2 M NaOH (10 min, 80ºC); LLE with isooctane | LOQ: 50 | 10 | [125] |
| COC, BZE, COE | Column-switching LC-ESI-MS/MS (QqQ) | Washing with DCM, MeOH; Extraction with 0.1 M HCl (overnight, 45ºC) | LOQ: 12-50 | 5 | [128] |
| COC, BZE, COE, NCOC, 6-AM, AP, MP, MDA, MDMA, MDEA, MTD | Nano-HPLC-Chip-ESI-MS/MS (QqQ | Washing with 0.1% SDS, water, acetone; Extraction with MeOH/ ACN/25 mM ammonium formate (25:25:50, v/v/v), (2 h at room temperature) | 0.1-0.75 | 2 | [129] |
| AP, MP, MDMA, MDEA, MBDB | LC-ESI-MS/MS (QqQ) | Decontamination with ethanol, DCM; Digestion with 1 M NaOH (15 min, 80ºC); LLE with hexane/ EtAc (2:1, v/v) | 0.3-6.3 | 20 | [87] |
| BZDs, hypnotics | LC-ESI-MS/MS (QqQ) | Washing with DCM (3 mL, 3 min, twice); Extraction with MeOH (overnight, 55ºC) | 0.2-4 | 100 | [113] |
| 18 opioids | LC-ESI-MS/MS (QqQ) | Washing with MeOH, water, MeOH; Extraction with MeOH (overnight) | 0.5-2.5 | 10 | [196] |
| Alprazolam, clobazam, DZP, clonazepam, zopiclone, midazolam, oxazepam, temazepam | LC-ESI-MS/MS (QqQ) | Washing with ethanol (3 mL, 5-10 min, twice); Extraction with MeOH/TFA (50:1, v/v, overnight) | 10-6,000 | 50 | [197] |

**Table 1.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| AP, MP, MDA, EDDP, MDMA, MDEA, COC, BZE, MOR, COD, heroin, 6-AM, MTD, THC, nicotine, cotinine, MPH | LC-ESI-MS/MS (QqQ) | Washing with DCM (3 mL, 10 min, 3 times); Pulverization and extraction with a ball mill (30 min) | LOQ: 30 | 50 | [198] |
| APs, BZDs, opiates, BZE, hallucinogens, antidepressants, THC, COC | LC-ESI-MS/MS (QqQ) | Washing with DCM (2 mL, 2 min, 3 times); Digestion with ACN (12 h, 50ºC); LLE with hexane and EtAc; SPE (reversed-phase cartridge, Strata X) | 0.2-50 | 50 | [109] |
| MOR, COD, 6-AM, COC, BZE | LC-ESI-MS/MS (QqQ) | Washing with DCM (1 mL, 3 times); Extraction with MeOH (4 h, 40ºC) | 1-10 | 20 | [199] |
| APs, BZDs, opiates, nicotine, cotinine, carisoprodol, meprobamate | LC-ESI-MS/MS (QqQ) | Washing with isopropanol, 0.01 M phosphate buffer, isopropanol; Extraction with ACN/25 mM FA (5:95, v/v), (overnight, 37ºC) | LOQ: 5-750 | – | [200] |
| THC, THC-COOH | LC-ESI-MS/MS (QqQ) | Washing sonication with MeOH; Extraction with 2.5 M NaOH at 60ºC; LLE with EtAc | 1 | 20 | [117] |
| APs, opiates, COC, BZE, EME, COE, NCOC, AME | LC-ESI-MS/MS (QqQ) | Washing with water, DCM; Incubation in phosphate buffer pH 5.0 (18 h, 45ºC); SPE (mixed-mode cation exchange, Oasis MCX) | 5-30 | 50 | [201] |
| AP, MP, MDMA, MDA, MOR, 6-AM, COD, COC, BZE, MTD, THC, DZP, 7-amino metabolites | LC-ESI-MS/MS (QqQ) | Extraction with ACN/MeOH/ 20 mM ammonium formate buffer pH 3 (10:10:80, v/v/v), (overnight, 37ºC) | 2-82 | 20 | [202] |

**Table 1.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| BZDs, zaleplon, zolpidem | LC-ESI-MS/MS (QqQ) | Washing with DCM (5 mL, 2 min, twice); Digestion in phosphate buffer at pH 8.4 (overnight); LLE with DCM and diethyl ether | LOQ: 0.5-5 | 20 | [119] |
| 18 BZDs | LC-ESI-MS/MS (QqQ) | Washing with DCM (5 mL, twice); Sonication in phosphate buffer pH 8.4 (1 h, room temperature); LLE with DCM | 0.2-5 | 20 | [70] |
| Scopolamine | LC-ESI-MS/MS (QqQ) | Washing with DCM (2 mL, 2 min, twice); Digestion in 0.01 M phosphate buffer at pH 8.4 (overnight); LLE with DCM: isopropanol:heptane (50:17: 33, v/v/v) | – | 50 | [203] |
| COC, APs, opiates, hallucinogens | LC-ESI-MS/MS (QqQ) | Washing phosphate buffer pH 6, isopropanol, DCM; PLE with water/MeOH (80:20, v/v), (100 bar; 150ºC; preheat time, 1 min; heat time, 7 min; static time, 5 min; flush volume, 0%; purge time, 60 s); SPE (reversed-phase cartridge, Strata X) | 0.5-4.7 | 100 | [78] |
| Mephedrone, 4-methyl-ephedrine, 4-methyl-norephedrine | LC-ESI-MS/MS (QqQ) | Washing with DCM (2 mL, 2 min, twice); Enzymatic digestion (2 h, 37.5ºC); LLE: for mephedrone with hexane, and for the metabolites with chloroform/ethanol/diethyl ether (3:1:1, v/v/v) | 2.5-5 | 50 | [133] |
| BZDs | LC-ESI-MS/MS (IT) | Washing with 0.1% SDS, water, DCM; Digestion with MeOH/ 25% ammonium hydroxide solution (sonicated 1 h and left at room temperature overnight); SPE (mixed-mode, Clean Screen®) | 1-20.7 | 30 | [112] |
| KET, NKET, AP, MP, MDA, MDMA | LC-ESI-MS/MS (IT) | Washing with 0.1% Tween 80, water; Extraction with 0.01% FA in an ultrasonic bath for 4 h | 100 | 20 | [111] |

**Table 1.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| KET, NKET | LC-ESI-MS/MS (IT) | Washing with 0.1% SDS, water, DCM; Extraction with MeOH/ 25% NaOH (20:1), (sonicated 1 h and left at room temperature overnight); MISPE | 100 | 10 | [86] |
| AP, MP | LC-ESI-MS/MS (IT) | Washing with 0.1% SDS, water, DCM; Extraction with 0.1 N phosphate buffer pH 5 (overnight, 45°C); MISPE | 30-780 | – | [85] |
| COC, BZE | LC-ESI-MS/MS (QqQ) | Washing with water, water/ MeOH mixture, DCM; Sonication in ACN (2 mL, 2 h); MISPE | 10-20 | 50 | [204] |
| MP, AP, MDA, MDMA, KET, NKET, COD, DHNK, 6-AM, MOR | LC-ESI-MS/MS (QqQ) | Washing with DCM (2 mL, 5 min); MAE with 0.25 N NaOH and MeOH/TFA (8.5:1.5, v/v), (3 min, 700 W) | 0.5-2.0 | 10 | [76] |
| EME, COC, BZE, MOR, 6-AM, COD, AP, MP, MDA, MDMA, EP, MDEA, MBDB, phentermine, phendime-trazine | LC-ESI-MS/MS (IT) | Washing with DCM (2 mL, 60 s, twice); Extraction with 0.1 N phosphate buffer pH 5 (overnight, 45°C); SPE (mixed-mode cation exchange, Bond Elute Certify) | 5-80 | 20-50 | [110] |
| 35 psychotropic drugs | LC-ESI-MS/MS (QqQ) | Washing with water, DCM; Incubation in phosphate buffer pH 5.0 (16 h, room temperature); LLE with DCM/ether (70:30, v/v) | 0.5-10 | 50 | [205] |
| THC-COOH, THC, CBN, CBD | LC-ESI-MS/MS (QTrap) | Washing with water, acetone, petroleum ether; Extraction with MeOH at room temperature | LOQ: 2.5-20 | 50 | [179] |
| BZDs, zaleplon, zolpidem, zopiclone | LC-ESI-MS/MS (QTrap) | Washing with water, acetone, hexane; Extraction with MeOH at room temperature (90 min) | – | – | [127] |

**Table 1.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| BZDs, zolpidem | LC-ESI-MS/MS (QTrap) | Extraction in MeOH (overnight, 38ºC) | 0.5-50 | 10-30 | [206] |
| 22 synthetic cannabinoids | LC-ESI-MS/MS (QTrap) | Washing with water, acetone, petroleum ether; Ultrasonication in ethanol (1.5 mL, 3 h) | 0.5-50 | 50 | [132] |
| BZP, mCPP, TFMPP, 4-FA, cathinone, KET, methcathinone, butylone, MDPV, methylone, mephedrone, ethylone, MPH | LC-ESI-MS/MS (QTrap) | Washing with water, acetone, hexane; Extraction in two steps, first ultrasonication in MeOH (5 mL, 16 h) and then ultra-sonication in MeOH/HCl 33% (3 mL, 3 h) | 10-50 | 20-30 | [130] |
| Opiates, APs, COC, BZDs, antidepressants, hallucinogens | HPLC-ESI-HRMS (LTQ-Orbitrap) | Washing with 10% SDS, water, acetone; Micropulverized extraction with water/ACN/1 M TFA (80:10;10, v/v/v), (10 min); Filtration using an Acrodisc with GHP membrane | 30-150 | 2.5 | [81] |
| BZDs | HPLC-ESI-HRMS (LTQ-Orbitrap) | Washing with isooctane, acetone; Incubation in phosphate buffer pH 8.4 (overnight, 37ºC); LLE with DCM/diethyl ether (9:1, v/v) | 0.5-10 | 50 | [102] |
| KET, NKET | LC-ESI-HRMS (LTQ-Orbitrap) | Washing with DCM, acetone; Micropulverized extraction with 1 M TFA (5 min); Filtration through a PTFE filter | 20 | 2 | [82] |
| AP, MP, MDA, MDMA, BZE, DMA, COC KET, NKET | LC-ESI-HRMS (LTQ-Orbitrap) | Micropulverized extraction in aqueous medium (5 min); Filtration through a 0.45 μm PVDF filter | LOQ: 50-500 | 0.2 | [83] |
| Opiates, cocainics, cannabinoids, APs | LC-ESI-MS (TOF) | Washing with shampoo, with water, acetone; Extraction with MeOH (sonicated 8 h at 50ºC and left at room temperature overnight); Filtration through a 0.45 μm PTFE | 5-75 | 20 | [100] |

**Table 1.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| AP, MP, COC, BZE, MDEA, COD, 6-AM, chlordiazepoxide, 7-amino metabolites, lorazepam, MDMA, MTD, 52 pharmaceuticals | UPLC-ESI-MS (TOF) | Extraction with MeOH/ACN/2 mM ammonium formate containing 8% ACN pH 5.3 (25:25:50, v/v/v), (overnight, 37ºC); Filtration through a PTFE filter | 10-100 | 10 | [99] |
| BZDs | UHPLC-ESI-MS (TOF) | Washing with 0.1% SDS, water, MeOH, water; MAE with EtAc (10 min, 75ºC, 1600 W) | 3-25 | 45 | [75] |
| Antidepressants, antipsychotics, antiepileptics, APs, opiods, β-blockers, alprazolam, zolpidem, metoclopramide, lidocaine, paracetamol | LC-ESI-MS (TOF) | Washing with ethanol (5 mL, 10 min); Digestion with 1 M NaOH (1 h, 100ºC); SPE (Isolute HCX-5) | – | 150-250 | [101] |
| 24 illicit drugs, BZDs | LC-ESI-MS (QTOF) | Washing with water, acetone; Two times extraction by incubating with MeOH/ACN/water/2 mM ammonium formate (25:25:50, v/v/v), (overnight, 37ºC) | 3-15 | 20 | [207] |
| 50 different new psychoactive drugs | LC-ESI- MS (QTOF) | Incubation in 0.5 M NaOH (overnight, 45ºC); LLE with hexane/EtAc (9:1, v/v) | LOQ: 10-20 | 100 | [131] |

abuse in hair samples due to the high signal-to-noise ratio and selectivity offered. LC coupled with time-of-flight (TOF) MS [99–101] or hybrid linear ion-trap-Orbitrap (LTQ-Orbitrap) MS [81–83, 102], which offer the advantage of accurate mass measurement, have also been successfully reported in the literature. However, LC–MS/MS is not free from drawbacks, the most important probably being its susceptibility to matrix effects, especially when applying ESI [103, 104]. Matrix effects can cause both higher signals (ion enhancement) and lower signals

(ion suppression) of the analytes, considerably affecting parameters such as limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision [96]. That said, the evaluation of possible matrix effects must be an integral part in any LC–MS/MS method development and validation [105]. Detailed descriptions on matrix effects have already been discussed elsewhere, as well as strategies to avoid them [106, 107]. The use of stable isotope-labelled internal standards (e.g. deuterium labelled compounds) serves to compensate for matrix effects [108]. When the cost of these labelled compounds is prohibitive, for example in multi-analyte procedures, or they are not commercially available, some groups have reported the use of the analogous compounds as internal standards [81, 99, 109].

With regard to the types of mass analysers used in LC–MS/MS, triple quadrupole (QqQ) instruments operating in selected reaction monitoring (SRM) mode are currently the most commonly used in drug analysis in hair, although ion trap (IT) instruments have also been proposed for this task [85, 86, 98, 110–112]. Recently, Bucelli *et al.* [110] developed and validated a LC–ESI-(IT)MS/MS method for the analysis of 16 drugs (COC and its metabolites, opiates and some stimulants) in human hair. From 20 to 50 mg aliquots hair, LOD values in the range 5–80 pg/mg were obtained, which are below those recommended by the SoHT [19]: 200 pg/mg for opiates; 500 pg/mg and 50 pg/mg for COC and its metabolites, respectively; and 200 pg/mg for APs. In particular, the advantages of using IT were greater sensitivity, good mass resolution and scan speed. The IT mass spectrometer made it possible to work over the entire mass range in full-scan mode, in MS/MS and $MS^n$ mode. The qualitative and quantitative method proved to be suitable for routine use in the anti-doping laboratory, as it was shown to be specific, accurate and precise across the calibration range.

The use of IT has been also reported by Klys *et al.* [98] who developed a method to screen eleven illegal and therapeutic drugs, including opiates, amphetamines (APs) and cocainics in human hair samples. A satisfactory chromatographic separation of the investigated drugs of abuse was achieved, which made it possible to monitor identical fragmented ions (e.g. ions-268 produced in the course of disintegration of MOR and 6-AM). The authors reported that the hair extracts obtained were characterized by a rich biological matrix, the effect of which was attenuated using the

MS/MS option in a quadrupole ion trap detector (QTrap). The use of this mode allowed concentration levels to be achieved for the analysed drugs of 50 pg/mg for cocainics, and 200 pg/mg for opiates and APs, which are in agreement with those recommended by the SoHT [19].

Many hypnotic drugs are effective agents for drug-facilitated sexual assaults, due to their wide and easy availability, low efficacious dose, and various pharmaceutical forms, facilitating clandestine administration. In this respect, a specific LC–ESI-(QqQ) MS/MS procedure for determining BZDs and hypnotics at low concentrations in hair was developed and fully validated [113]. The LODs achieved ranged from 0.2 pg/mg for KET to 4.0 pg/mg for flunitrazepam. Matrix effects were within acceptable ranges, as well as accuracy, repeatability and precision. Often, two ion transitions in SRM mode are described as sufficient for correct identification of analytes [114]. However, cases are described in which two ion transitions were not sufficient for correct identification [115, 116]. Using three ion transitions, as in the proposed method, provides a high level of certainty in identification, even after a single drug intake, making it very useful in cases involving drug-facilitated crimes.

The detection of cannabinoids in hair is a great analytical challenge since they are usually found at very low concentrations (in the pg/mg range). In this context, Mercolini *et al.* [117] developed and validated a method for the determination of $\Delta^9$-tetrahydrocannabinol (THC) and its metabolite, THC-COOH, in hair samples using a LC–ESI-(QqQ) MS/MS system. A LOD of 1 pg/mg for both analytes was reported. The SoHT recommended a cut-off of 50 pg/mg for THC and one of 0.2 pg/mg for THC-COOH as suitable levels for detecting occasional users and for confirmation analyses. Due to the weak incorporation of THC-COOH into the hair matrix, its concentration in hair is even lower than the concentration of the parent drug. This means that using the proposed method in this study might lead to false-negative results for marijuana use, which is especially significant if there are legal implications of drug consumption.

Owing to the tendency for polydrug consumption, multi-analyte methods are recommended, saving time, costs and amount of sample required. In this regard, Lendoiro *et al.* [109] established an LC–ESI-(QqQ) MS/MS multiclass screening method for the simultaneous detection of 35 licit and illicit drugs and metabolites in hair. For the entire range of investigated compounds, the LODs

were 0.2–50 pg/mg, which are lower than the cut-off values reported in the SoHT guidelines [19]. The authors reported that 31 out of 35 analytes showed considerable matrix effect: 27 showed ion suppression and 4 displayed ion enhancement. In all cases % coefficient of variation (CV) was below 20%, except for AP, MP, 7-aminoflunitrazepam, methadone (MTD) and THC, with values up to 47.6%. Nevertheless, the use of the corresponding deuterated analogues for these analytes as internal standard compensated for these effects. One SRM transition per compound was monitored for the initial screening. To confirm a positive result, a second injection was performed monitoring two transitions per compound. Thus, the present protocol may be suitable for easy application in routine analysis for toxicological investigations.

Another highly sensitive LC–ESI-(QqQ)MS/MS method for the determination of fourteen different drugs of abuse in human hair samples was recently published, providing LOD values between 0.5 and 4.7 pg/mg [78]. The proposed analytical procedure showed ion suppression always by less than 14%, except for PCP, for which sensitivity was reduced by 29%, but the use of PCP-d5 as internal standard compensated for this effect. Accuracy, precision, linearity, sensitivity and specificity were satisfactory. Therefore, the method seems to be suitable for routine determination of the tested drugs in hair. Unfortunately, in order to cope with 14 compounds at low pg/mg quantities in hair, the amount of sample could not be reduced to less than 100 mg, which may be a drawback when the sample is limited.

High-resolution mass spectrometry (HRMS) is a powerful tool due to the possibility of performing full-scan analysis and accurate mass measurements, allowing the identification and elucidation of unexpected compounds, metabolites and degradation products. Currently, the main HRMS techniques are LTQ-Orbitrap and TOF mass spectrometers. For instance, a fully validated method capable of the detection and confirmation of 28 BDZs in 50 mg of hair using LC-ESI-HRMS has been described [102]. The high sensitivity and specificity required for the detection of BZDs at pg/mg levels in hair samples were obtained by the use of a LTQ-Orbitrap mass spectrometer, achieving LODs from 0.5 to 10 pg/mg in this way. Matrix effects led to significant ion suppression for the majority of BDZs, mainly for 7-aminonitrazepam and 7-amino-clonazepam (the two analytes at the lowest retention time, eluting among the most polar compounds).

However, the use of the corresponding deuterated internal standards compensated for these effects. The Orbitrap mass spectrometer uses non-specific fragmentation, rather than multiple mass spectrometry on preselected ions, allowing one to work in full-scan mode, without splitting the acquisition segment. In this way, the accurate mass of all ionizable compounds eluting in the HPLC run are stored and a "retrospective" screening can be carried out, searching the raw data for new metabolites or other drugs suspected at a later time. The described method is appropriate for both the screening and confirmation of the target compounds and allows the determination of single doses in forensic toxicology cases, as well as documenting chronic exposures.

Later, the same group developed and validated an analogue method to detect 28 compounds that belonged to several drug classes, such as COC, APs, opiates, BZDs, antidepressants and hallucinogens [81]. A mass spectrometric approach similar to the one previously described was applied in conjunction with a new and faster extraction process based on micropulverization extraction of the hair, providing LOQs between 100 and 500 pg/mg, which are particularly suitable for monitoring therapeutic administration or for forensic determination of the target drug classes. The proposed method allows reliable identification and quantification of the target compounds using only 2.5 mg of hair sample. Reducing the amount of hair required is beneficial to both investigators and suspects. Calibration curves were generated in the range from LOQ to 25,000 pg/mg and 50,000 pg/mg. The coefficient of determination ($r^2$) was >0.99 for all the analytes using a 1/x weighted linear regression, except for APs, for which a quadratic response was observed. The use of quadratic models is recommended if the accepted criteria are not satisfied with the linear model. The authors pointed out that a possible explanation of the present lack of linear fit could be the use of methylendioxypropylamphetamine (MDPA) as internal standard. As discussed, full-scan HRMS Orbitrap allows for expansion of the monitoring program when the presence of a new drug is detected or for support the presence of parent drugs by retrospective analysis of untargeted metabolites. In this respect, the authors reported that, with this approach, COC metabolites such as cocaethylene (COE) and norcocaine (NCOC), levamisole (adulterant of street COC), haloperidol, venlafaxine metabolites

or BZDs not included on the list of 28 compounds have been identified, among others, in real samples. Nevertheless, at the present time, the high capital cost of HRMS Orbitrap instruments limits their widespread use.

Meanwhile, TOF instruments are more affordable and have been shown to be useful for the determination of multiclass drugs of abuse in hair samples [75, 99–101]. UHPLC–ESI–TOF–MS screening procedure targeted 52 common drugs and pharmaceuticals in hair samples [99]. The total run-time was 17 min. For the entire range of investigated compounds, LODs were between 10 and 100 pg/mg, which are appropriate for the screening of pharmaceuticals and drugs in human hair. For most of the analytes, the LOQ was 50 pg/mg. However, for AP, MDA, MDMA, MP and MOR the LOQ did not reach the cut-off values (200 pg/mg) recommended by the SoHT. The authors underlined that the obtained LOD and LOQ values in this paper are slightly higher that the values obtained with existing LC–MS/MS methods [98, 118, 119]. However, all these reported methods have been restricted to drugs of abuse or limited drug groups. The developed TOF method has the advantage of being able to analyse 52 multi-target drugs simultaneously. Furthermore, the TOF instrument gives the option of working with larger libraries compared to LC–MS/MS instruments, which makes this approach extremely good for screening purposes of unknown analytes. According to the authors, the sensitivity of the developed method can be improved by introduction of a concentration step.

Hydrophilic interaction liquid chromatography (HILIC) has become an alternative to conventional RP HPLC as it is more convenient for the analysis of highly polar compounds that are weakly retained or eluted with the dead volume in conventional RP HPLC. In this regard, to detect COC and all its metabolites in hair, including the highly polar ecgonine methyl ester (EME), HILIC has been proposed in the literature [120]. After acidic incubation and SPE, the extracts were injected into a HILIC system interfaced to a QqQ mass spectrometer with an ESI source. The method proved to be highly sensitive, providing a LOD of 1 pg/mg for COC, benzoylecgonine (BZE) and EME, and of 0.5 pg/mg for COE. This is a significant achievement, especially for EME, a potential marker for the ingestion of COC, since this metabolite occurs at very low levels in hair and its analysis is particularly challenging using standard

RP-HPLC due to its poor retention.

New and sophisticated methods continue to emerge, such as the method based on the use of instruments and analytical columns that tolerate high pressures, known as fast LC, ultra-high performance LC (UHPLC), or ultra-performance LC (UPLC) coupled to QqQ or TOF analysers [75, 99, 100, 121–125]. UHPLC systems use stationary phases with particle sizes of around two micrometres or even smaller, improving chromatographic performance in comparison with methods based on conventional HPLC. Due to the higher separation power of such columns, multiple analytes can be separated in comparatively short run-times.

Di Corcia *et al.* [121] achieved the separation of thirteen common drugs of abuse and metabolites within 5.5 min plus 2.5 min of column re-equilibration time. The analytes were extracted from the hair matrix by overnight incubation with an organic solvent and then the extracts were directly injected into a UHPLC–ESI-(QqQ)MS/MS system. It is well known that a major drawback of LC–MS/MS procedures with direct injection of the sample is the susceptibility to matrix effects. However, in this work, the effect of real hair matrix did not appear significant for most of the analytes tested. The UHPLC generates narrow peaks, which reduces the likelihood of unwanted interferences. Accuracy, repeatability and precision were within acceptable ranges. The LOQs were below the cut-off values recommended by the SoHT [19] and ranged from 20 to 80 pg/mg. The use of UHPLC considerably reduces the overall time required for the analysis, without sacrificing the chromatographic resolution, nor the accuracy and precision for quantitative determinations, as well as low solvent use, making the described method quite suitable for routine analysis for toxicological investigations.

More recently, a multiclass screening method for the simultaneous identification and quantification of 96 drugs (from different groups: opiates, APs, hallucinogens, BZDs, antihistamines, anti- depressants, antipsychotics, barbiturates, muscle relaxants, etc.) in hair samples was proposed using UPLC–ESI-(QqQ)MS/MS system [122]. The run-time was 19.5 min, with all compounds eluting between 1.40 and 16.05 min. With 10 mg of decontaminated and overnight incubated hair, the experimental LOQs ranged from 2 to 50 pg/mg for basic analytes and from 100 to 5,000 pg/mg for acidic and neutral analytes. The low LODs achieved for some analytes may allow the

detection of a single drug exposure to these compounds, with possible application in drug-facilitated assault cases, e.g. KET and z-compounds (zolpidem, zaleplon, zopiclone, zonisamide). Matrix effects were within acceptable ranges, as well as accuracy, repeatability and precision. The present method allows the most comprehensive targeted screening of drugs in hair to date. Figure 3 shows the SRM chromatograms recorded from a blank hair sample spiked with all the analytes at 100 pg/mg for basic compounds and 10,000 pg/mg for acidic and neutral compounds. Due to the speed of analysis and low solvent use, UHPLC methods can be considered green approaches and very useful in laboratories where hundreds of samples are analysed per day.

Recent advances in HPLC column technologies include core–shell HPLC columns that contain a durable, homogenous porous shell which is grown on a solid silica core. This technology provides the efficiency of a column with smaller particle size without increasing the backpressure [126]. This allows toxicological laboratories to achieve the performance advantages of UHPLC methods on a traditional

HPLC without the need to invest in new capital equipment. For example, using a 2.6-micron core–shell HPLC column, 21 BZDs and 3 z-drugs were separated in less than 17 min [127]. Coupled with a QTrap mass spectrometer, target compounds were accurately quantified down to low concentration levels (LOQs between 0.6 and 16 pg/mg), making the proposed LC–ESI-(QTrap)MS/MS method a reliable means to screen for and confirm the presence or absence of the studied compounds.

New approaches for drug testing in hair have been proposed in the scientific literature, such as column-switching techniques, employed to elute the extracted analytes from a precolumn into a HPLC analytical column. This strategy was applied for the simultaneous determination of COC and its metabolites in only 5 mg of hair using a column-switching LC–ESI-(QqQ)MS/MS system [128], providing comparable results to GC–MS and allowing for a reduction in the sample amount of up to ten times. The use of an additional LC column increases sensitivity compared to a single LC column by reducing the introduction of interfering ions to the MS. Thus, good sensitivity was achieved, but low recoveries below 60% were obtained. The basic advantage of using column-switching is the potential savings in analysis time, since it provides the possibility of avoiding an extraction step. Additionally, selectivity can be

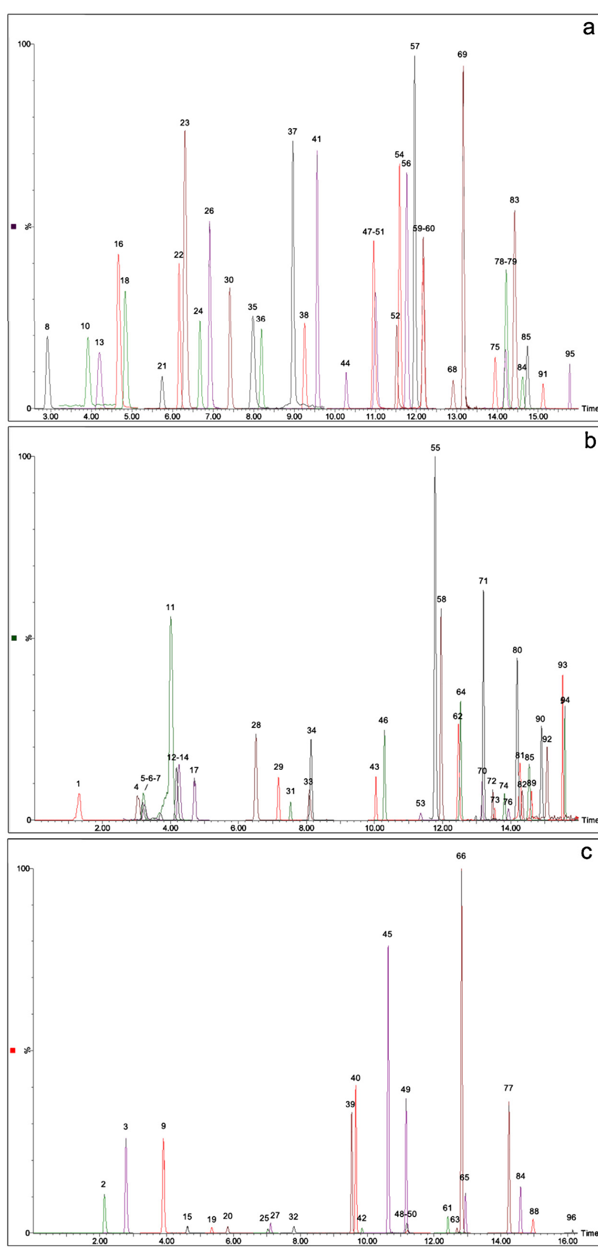



**Figure 3.** Selected reaction monitoring chromatograms recorded from a hair sample spiked with all analytes (basic compounds at 100 pg/mg, and acidic and neutral compounds at 10,000 pg/mg). For each analyte, only the quantifier transition is shown. (a, b) Basic compounds; (c) acidic and neutral compounds. Reproduced from [122] with the permission of Elsevier, copyright 2010.

improved by using a different type of precolumn to the analytical column. Nevertheless, the described method requires additional instrumentation.

Meanwhile, Zhu *et al.* [129] developed a microfluidic chip-based nano-HPLC-chip-ESI-(QqQ)MS/MS to analyse 14 drugs and metabolite in only 2 mg of hair. The very high sensitivity achieved (LOD and LOQ values were from 0.1 to 0.75 pg/mg and 0.2 to 1.25 pg/mg, respectively), as well as the low amount of hair sample used and the relatively short analysis time 2 h for the extraction of analytes from within the hair matrix plus 15 min for chromatographic separation), make this method appealing for the determination of drugs in hair for clinical diagnosis

Recent abuse of new designer drugs (e.g. synthetic cathinones, synthetic cannabinoids or piperizines) has presented a requirement for the introduction of sensitive, reliable and reproducible analytical methods for their determination in hair [39]. With this in mind, a number of screening and quantification methods using LC have been published [123, 124, 130–133].

A LC–ESI-(QTrap)MS/MS method was developed for screening new psychoactive substances in hair [130]. Hair samples that were tested positive for APs or MDMA (N=325) were reanalysed concerning novel drugs of abuse such as cathinones [methylone, butylone, ethylone, methylenedioxy-pyrovalerone (MDPV), mephedrone, methcathinone and cathinone], piperizines (BZP, TFMPP and mCPP), 4-fluoroamphetamine (4-FA), methylphenidate (MPH) and KET. A two-step extraction procedure was carried out. The LODs achieved were 50 pg/mg for cathinone and 10 pg/mg for the remaining drugs. Using three ion transitions per analyte followed by an enhanced product ion scan as dependent scan, which gives spectral information, provides a high level of certainty in identification that can easily withstand forensic scrutiny. In Figure 4 is reported the SRM chromatogram of 4-FA and mCPP indicating the presence of these 2 drugs in a real hair sample, and the corresponding enhanced product ion spectrum of mCPP.

Salomone *et al.* [123] established an UHPLC–ESI-(QqQ)MS/MS method for the simultaneous determination of 23 synthetic cannabinoids. The whole chromatographic run was completed within 9 min, with all compounds eluting between 2.2 and 5.5 min. The LOQ values lied between 0.7 pg/mg and 4.3 pg/mg (80 pg/mg for HU-210). The present method proved to be simple, fast, specific and sensitive. Unfortunately, since the number of synthetic cannabinoids compounds

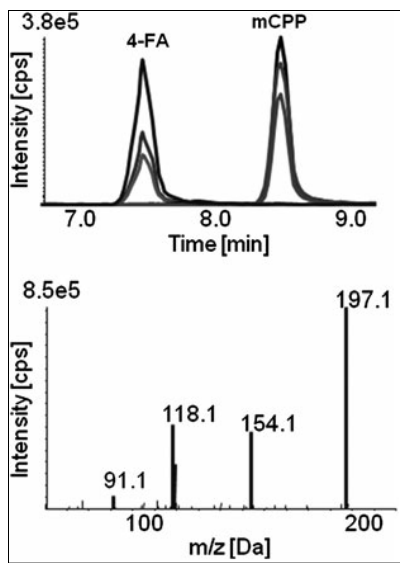



**Figure 4.** Extracted ion chromatograms of the Selected Reaction Monitoring transitions recorded from a real hair sample of 4-fluoroamphetamine (4-FA, 154/ 109, 154/137, 154/83) and metachloro-phenylpiperazine (mCPP, 197/154, 197/118, 197/119) (upper) and the corresponding enhanced product ion-spectrum of mCPP (lower part). Reproduced from [130] with the permission of Elsevier, copyright 2010.

on the market appears to grow every month, their identification is extremely difficult, particularly because pure standards as well as fragmentation spectra are hardly available [39, 134]. In this respect, a LC–ESI-QTOF–MS method, based on accurate mass and isotopic pattern recognition, was successfully applied for a broad-spectrum toxicological analysis of hair [131]. The approach proved to be suitable not only for searching of synthetic cannabinoids but also of other new designer drugs, such as cathinones, phenylethyl-amines and piperizines.

*4.2. Gas chromatography*

GC–MS is another very commonly used technique in drugs of abuse analysis in hair samples, as can be seen in Table 2, with electron impact (EI) ionization being the most widely used ion source, as it provides low LODs and also high specificity. However, in the analysis of drugs of abuse by GC, derivatization of the analytes is necessary prior to instrumental analysis and this additional step can be considered as a drawback since it clearly lengthens the analysis time and may complicate

the sample pretreatment. As a consequence, LC is normally the preferred choice, although interesting approaches using GC–MS can be found in the literature, since this alternative is cheaper than LC–MS/MS, and, therefore, is often used in testing laboratories for drugs of abuse.

A GC–EI/MS analytical method to simultaneously quantify APs, KET, opiates and metabolites in hair was proposed by Wu *et al.* [135]. In this paper, the samples, after extraction with an organic solvent, were subsequently cleaned up and preconcentrated by SPE (Bond Elut Certify) and then derivatized with heptafluorobutyric acid anhydride (HFBA). Despite the simple instrumentation used, the method proved to be sensitive and specific, providing LODs between 30 and 80 pg/mg using 25 mg of hair sample, which makes the method suitable for the simultaneous determination of a wide variety of drug classes in human hair. The simultaneous quantitative determination of several compounds types is advantageous because it allows hair analyses to be performed without the need of using several methods, since the majority of existing GC–MS methods have been limited to one or two drug groups. Thus, the described approach is beneficial to forensic and clinical toxicologists.

Another interesting approach based on GC–EI/MS was the reported by Cordero and Paterson [136] who used a two-step derivatization in order to be enable the determination of a group of fourteen compounds [including APs, opiates, cocainics, diazepam (DZP) and metabolites] in hair samples. In this case, the authors used *N*-methyl-bis-trifluoroacetamide (MBTFA) and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchloro-silane (TMCS) as derivatizing agents, and they obtained LOQs of 100 pg/mg for APs and 200 pg/mg for the remaining drugs, using 50 mg of hair. With the exception of COC metabolites, these LOQ values are in agreement with the cut-off suggested by the SoHT [19]. This means that using the proposed method in this study might lead to false-negative results for COC intake, which is especially significant if there are legal implications of drug consumption. Despite of the simplicity of the used instrumentation and the reasonably promising results obtained in terms of analytical precision [relative standard deviations (RSDs) <25% for real samples in both intra-day and day-to-day experiments], the use of a double derivatization step

**Table 2.** Gas chromatography-based methods for the determination of drugs of abuse in hair samples.

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| COC, BZE | GC-EI/MS | Washing with DCM, water, MeOH; Extraction with MeOH and 0.1 M HCl (3 h, 65ºC); SPE (mixed-mode cation exchange, Oasis MCX); Derivatization with MSTFA + 5% CTMS (25 min, 80ºC) | 15-20 | 20 | [181] |
| MOR, COC | GC-EI/MS | Washing with DCM, water, MeOH; Extraction with MeOH in 0.1 M HCl (overnight, 65ºC); SPE (mixed-mode cation exchange, Oasis MCX); Derivatization with MSTFA (30 min, 80ºC) | 10-20 | 20 | [208] |
| AP, MP, MDA, MDMA, MDEA, KET, NKET, MOR, COD, 6-AM | GC-EI/MS | Washing with DCM (2 mL, 5 min); Extraction with MeOH/TFA (overnight, 25ºC); SPE (mixed-mode cation exchange, Bond Elut Certify); Derivatization with HFBA (30 min, 70ºC) | 30-80 | 25 | [135] |
| COC, BZE, COE, heroin, 6-AM, MOR, COD, 6-acetylcodeine (6-AC) | GC-EI/MS | Washing with water, petroleum benzene, DCM; Digestion by ultrasonication with MeOH (5 h, 50ºC); SPE (mixed-mode cation exchange); Derivatization with MSTFA (15 min, 90ºC) | 10-50 | 50 | [68] |
| MOR, 6-AM, COD, 6-AC, tramadol | GC-EI/MS | Washing with DCM, water, MeOH; Extraction with MeOH (overnight, 65ºC); SPE (mixed-mode cation exchange, Oasis MCX); Derivatization with MSTFA + 5% TMS (25 min, 80ºC) | 50 | 20 | [180] |
| MOR, 6-AM, COD, MDA, HCOD, COC, BZE, EME, COE, DZP, NDZP, MP, MDMA, | GC-EI/MS | Washing with DCM, isopropanol, acetone; Digestion with 0.1 M HCl (overnight, 50ºC); SPE (mixed-mode cation exchange, Bakerbond Narc-2); Two-step derivatization with MBTFA and MSTFA + 1% TCMS (80ºC, 30 min) | LOQ: 100-200 | 50 | [136] |

**Table 2.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| AP, MP, MDA, MDMA | GC-EI/MS | Washing with diluted soap solution at physiological pH, water; Digestion with 1 M NaOH (70ºC, 30 min); LLE with chloroform; Microwave derivatization with MBTFA (3 min, 300 W) | 20 | 20 | [209] |
| TFMPP, mCPP, 1-(4-methoxy-phenyl) piperazine | GC-EI/MS | Washing with DCM, water, MeOH; Digestion with 1 M NaOH (40 min, 50ºC); SPE (mixed-mode cation exchange, Oasis MCX); Derivatization with MSTFA + 5% TMS (30 min, 80ºC) | LOQ: 50 | 20 | [146] |
| COC, BZE, COD, MOR, 6-AM | GC-EI/MS | Washing with diluted soap solution at physiological pH, water; Enzymatic ultrasonication (30 min, 30ºC); SPE (reversed-phase cartridge, Oasis HLB); Derivatization with BSTFA + 1% TMCS (20 min, 100ºC) | 20-50 | 50 | [210] |
| COC, THC | GC-EI/MS | Washing with DCM (3 mL); Enzymatic digestion (50 min, 37.5ºC); LLE with pentane | 15-20 | 50 | [211] |
| COD, MOR, 6-AM | GC-EI/MS | Extraction with MeOH (overnight, 56ºC); Derivatization with BSTFA + 1% TMCS; HS-SPME (PDMS, 100 μm) | 2-5 | 10 | [90] |
| COC, BZE, COE | GC-EI/MS | Washing with a 0.1 % solution of Tween 80, water; Enzymatic digestion (2 h, 37ºC); SPE (reversed-phase cartridge, Oasis HLB); Derivatization with BSTFA + 1% TMSC (20 min, 100ºC) | 10-40 | 50 | [161] |
| AP, KET, MTD, COC, COE, THC | GC-EI/MS | Washing with water, acetone; Digestion with 1 M HCl (60 min, 60ºC); For THC, digestion with 1 M NaOH; Derivatization with MSTFA; HS-SPME (PDMS, 100 μm; 10 min, 90ºC) | 10-120 | 10 | [88] |

**Table 2.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| THC, CBD, CBN | GC-EI/MS | Washing with water, acetone; Digestion with 1 M NaOH (20 min, 80ºC); Derivatization with BSTFA + 1% TMCS; HS-SPME (PDMS, 100 μm) | 10-20 | 15-30 | [94] |
| THC, CBD, CBN | GC-EI/MS | Washing with petroleum ether (2 mL, 10 min, 3 times); Digestion with 1 M NaOH (20 min, 90ºC); HS-SPME (PDMS, 100 μm) | 70 | 10 | [93] |
| AP, MP, MDA, MDMA, COD, 6-AM, COC, BZE, COE, NCOC, MTD, oxycodone, oxymorphone, hydrocodone, meperidine, hydro-morphone | GC-EI/MS | Washing with DCM; Extraction with MeOH (overnight, 56ºC); SPE (mixed-mode cation exchange, Oasis MCX); Derivatization with BSTFA-MSTFA + 1% TMCS; HS-SPME (PDMS, 100 μm) | 130-200 | 10 | [91] |
| AP, MP, fenproporex, MDMA, MDA | GC-EI/MS | Washing with DCM (2 mL, twice); Incubation in 1 M NaOH (15 min, 70ºC); HF-LPME (9-cm fibre, solvent extraction: dihexyl ether, acceptor phase: 0.1 M HCl) | LOQ: 50 | 50 | [95] |
| AP, MP, MDMA, MDA | GC-EI/MS | Digestion with 5 M NaOH (10 min, 80ºC); HS-SPME (PDMS, 100 μm; 10 min, 80ºC) | 10-500 | 10 | [92] |
| COC, BZE, COD, MOR, 6-AM | GC-EI/MS | Washing with diluted soap solution at pH ~ 7.4, water; MSPD on column SPE (reversed-phase cartridge); Derivatization with BSTFA + 1% TMCS (20 min, 100ºC) | 20-50 | 50 | [79] |
| COC, BZE, COD, MOR, 6-AM | GC-EI/MS | Washing with diluted soap solution at physiological pH, water; MSPD-enzymatic hydrolysis; SPE (reversed-phase cartridge, Oasis HLB); Derivatization with BSTFA + 1% TMCS (20 min, 100ºC) | LOQ: 40-180 | 50 | [72] |

**Table 2.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| AP, MP | GC-EI/MS | Micropulverized extraction in aqueous medium (5 min); Aqueous acetylation (20 min, room temperature); MEPS ($C_{18}$) | LLOQ: 200 | 1-5 | [84] |
| AP, MP, MDA, MDMA, NKET | GC-EI/MS | Washing with water, acetone; Pulverization and ultrasonication with MeOH (1 h, 50ºC); Filtration through a 0.2 μm PTFE filter; Derivatization with HFBA (30 min, 50ºC) | 28 | 10 | [69] |
| THCA-A, THC | GC-EI/MS | Washing with water, acetone, petroleum ether; Digestion with 1 N NaOH (10 min, 95ºC); LLE with hexane/EtAc (9:1, v/v); Derivatization with MSTFA | 20-50 | 30-50 | [35] |
| Phentermine | GC-EI/MS | Extraction with 1% HCl in MeOH (20 h, 38ºC); Derivatization with TFA/EtAc (1:1, v/v) | 100 | 10 | [213] |
| Diazepam, lorazepam, midazolam, zolpidem | GC-EI/MS | Washing with water, MeOH; Incubation in MeOH (16 h, 38ºC); SPE (mixed-mode); Derivatization with MSTFA (20 min, 90ºC) | 160-330 | 30 | [212] |
| Mephedrone | GC-EI/MS | Washing with DCM (2 min, room temperature); Incubation in Soerensen buffer pH 7.0 (overnight, 40ºC); LLE with EtAc; Derivatization with HFBA | LOQ: 200 | 50 | [145] |
| AP, MP, MDA, phentermine, cathinones, methcathinone, fenfluramine, desmethyl-selegiline, MDMA, MDEA, NKET, mescaline, 4-bromo-2,5-dimethoxy-phenethylamine | GC-EI/MS | Washing with water, acetone; Ultrasonication in 0.25 M HCl (1 h, 50ºC); Derivatization with TFAA (30 min, 70ºC) | 2-24 | 20 | [147] |

**Table 2.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| COC | GC-EI/MS | SFE procedure | 100 | 25 | [77] |
| AP, MP, MDA, MDMA, MDEA, KET, NKET, MOR, COD, 6-AM | GC-EI/MS GC-NCI/MS | Washing with DCM; Extraction with MeOH/TFA (overnight, 25ºC); SPE (mixed-mode cation exchange); Derivatization with HFBA (30 min, 70ºC) | 0.025-10 | 25 | [137] |
| THC, CBD, CBN | GC-EI-MS/MS (IT) | Washing with petroleum ether, water, DCM; Digestion with 1 M NaOH (15 min, 90ºC); HS-SPME (100 μm PDMS, 40 min, 90ºC) | 7-31 | 10 | [89] |
| COC, AEME, EME, COE | GC-CI-MS/MS (IT) | Washing with DCM, water, MeOH; Incubation in 0.1 M HCl (overnight, 60ºC); Automated SPE | 5-50 | 50 | [141] |
| Methyl-testosterone, nandrolone, boldenone, fluoxy-mesterolone, COC, BZE | GC-EI-MS/MS (IT) | Washing with DCM; For anabolic steroids: Extraction with 1 M NaOH (15 min, 95ºC); SPE ($C_{18}$); LLE with diethyl ether; Derivatization with MSTFA-$NH_4I$-DTE (30 min, 70ºC); For COC and BZE: Extraction with MeOH (2 h, 56ºC) Derivatization with MSTFA-$NH_4I$-DTE (30 min, 70ºC) | 10-100 | 50 | [138] |
| THC-COOH | GC-NCI-MS/MS (QqQ) | Washing with isopropyl alcohol (10 mL, 3 times); Incubation in 1.0 M NaOH (30 min, 95ºC); LLE with hexane/EtAc (9:1, v/v); Derivatization with PFPA/ PFPOH | 0.02 | 25 | [139] |
| THC-COOH, THC | GC-NCI-MS/MS (QqQ) | Digestion with 1 M NaOH (15 min); LLE with hexane/EtAc (9:1, v/v); Derivatization with PFPA + HFIP | 0.01 | 20-50 | [140] |
| Fentanyl, carisoprodol, oxycodone, propoxyphene, tramadol, DZP, MTD, MOR | 2D GC EI/MS | Washing with acetone; Sonication with 25 mM phosphate buffer (pH 2.7); SPE (mixed-mode cation exchange, Cerex Clin II) | LOQ: 5 | 10 | [71] |

**Table 2.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| THC-COOH | 2D GC-NCI-MS | Washing with DCM (1.5 mL); Extraction with 2 N NaOH (15 min, 75ºC); SPE (mixed-mode cation exchange, Bond Elut Certify); Derivatization with TFAA + HFIP | 0.05 | 20 | [143] |
| COD, MOR, 6-AM, AP, MP, MDA, MDMA, MTD, BZP | 2D GC-TOF-MS | Washing with DCM (1 mL, 3 times); Extraction with 0.1 M HCl (overnight, 37ºC); SPE (mixed-mode cation exchange, Bond Elut Certify); Derivatization with MTBSTFA + 1% TBMCS | – | 20 | [144] |
| AP, MP | GC-HRMS | Washing with water, acetone; Extraction with MeOH/5 M HCl (20:1, v/v) (ultrasonicated 1 h and left at room temperature overnight); Filtration and derivatization with TFAA | 9-21 | 30 | [142] |

compromises the overall analysis time and, therefore, the method is not really suitable for routine hair analysis.

In general, most of the reported GC–EI/MS methods to date have employed a SPE procedure for cleaning up of the sample and the preconcentration of the analytes, despite the fact that it requires relatively large volumes of solvents. In line with the trend towards environmentally friendly procedures, SPME has emerged as a versatile solvent-free alternative to conventional SPE, and gained considerable interest in a wide field of analysis including drug abuse. SPME in HS mode has been used in conjunction with GC–MS system for the analysis of a variety of drugs of abuse analysis of a variety of drugs of abuse in hair samples [88–94].

Merola *et al.* [88] reported a GC–EI/MS method using SPME with on-fibre derivatization of APs, KET, MTD, COC, COE and THC in 10 mg of hair. They used a polydimethylsiloxane (PDMS)-coated fibre of 100 mm thickness and MSTFA as the derivatizing agent. The LOQs were below the cut-off recommended by the SAMHSA [20], except for AP and COE, and ranged from 20 to 370 pg/mg. The high sensitivity achieved for THC (LOD

10 pg/mg and LOQ 20 pg/mg) makes the method suitable for detecting occasional cannabis use. Nevertheless, the fact that authors used alkaline digestion for THC isolation, as well as acidic extraction for the remaining drug isolation from the hair matrix makes the proposed method time-consuming and, therefore, not really suitable for routine hair analysis. Furthermore, the sensitivity of this method may be affected by certain problems regarding the HS-SPME procedure, such as carryover, contamination, handling of the procedure, and life time of fibre. In particular, when the SPME fibre is dipped into or exposed to a derivatizing reagent, the fibre coating could be damaged and, consequently, its lifetime is shortened.

In this context, an automated GC–EI/MS method was developed for the analysis of APs in human hair (10 mg) using in-matrix derivatization and SPME [92]. A PDMS-coated fibre of 100 mm thickness was used and ethylchloroformate was employed as the derivatizing agent in this work. In comparison with the findings of Merola *et al.* [88], the authors achieved better sensitivity for AP, MP and MDMA (LODs between 10 and 50 pg/mg), except for MDA (LOD 500 pg/mg). The established LODs are suitable for monitoring therapeutic levels of APs in human hair. This approach appears to be superior to the method described above, because damage to the SPME fibre is minimal and the fact of being automated shortens the overall analysis time (about 30 min per sample).

More recently, Aleksa *et al.* [91] published a GC–EI/MS method to detect 17 drugs of abuse in hair samples with the combination of a conventional SPE (Oasis MCX) followed by HS-SPME (PDMS-coated fibre 100 mm) after derivatization with a mixture of *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), MSTFA and TMCS. Although the protocol was expected to be more sensitive, LODs between 130 and 200 pg/mg were achieved for the tested drugs, which are particularly suitable for monitoring therapeutic administration or for forensic determination. However, the additional evaporation step after the SPE procedure makes the method quite time-consuming and laborious and, consequently, not entirely suitable for routine hair analysis.

Hollow-fibre liquid-phase micro-extraction (HF-LPME) is another relatively new miniaturized technique that has gained considerable interest in a broad field of analysis, including drugs of abuse, mainly due the low consumption of organic solvents (low microlitres range). A new method

based on this approach and GC–EI/MS was developed for the determination of AP-type stimulants in samples of human hair [95]. A 9-cm hollow fibre, filled with dihexyl ether in its pores, was used for extraction. The fibre lumen was filled with acceptor phase [1 M hydrochloric acid (HCl)] and introduced into the sample solution. This method proved to be suitable for the separation of primary and secondary amines, reproducible (RSDs <11% for all APs, in both intra-day and day-to-day experiments) and highly sensitive and specific (LOQs for all APs around 50 pg/mg, which are below the cut-off value established by the SoHT [19]). In contrast to the SPME fibre, the hollow-fibre can be discarded after each extraction due to its low cost, thus eliminating any possibility of carryover.

Miyaguchi *et al.* [84] developed a simple and fast (1 h) method for sample preparation named MiAMi (micropulverized extraction-aqueous acetylation-MEPS) followed by GC–EI/MS analysis for the rapid identification and quantification of MP and AP in hair samples. The amount of hair sample required for qualitative analysis based on full-scan mass spectra was only 5 mg, whereas, 1 mg of hair sample was sufficient for quantification analysis, making it a very useful method when sample availability is limited. Later, a faster (30 min) drug isolation, clean-up and preconcentration process based on MSPD and SPE was coupled with GC–EI/MS analysis using a mixture of BSTFA and TMCS as the derivatization agent [79]. The method allowed reliable identification and quantification of five basic abuse drugs [COC, BZE, codeine (COD), MOR and 6-AM] using 50 mg of hair sample, providing LODs between 20 and 50 pg/mg, which are better than cut-off values reported in the SoHT guidelines, offering good analytical precision with RSDs 13% in real samples, in both intra-day and day-to-day experiments. Both described methods, [84] and [79], are promising strategies that should be further applied to assess other drugs.

In some cases, chemical ionization (CI) mode has also been used because this technique may provide more selectivity, as it often gives molecular mass information, whereas the EI mode provides more structural information. In this regard, sensitivity can be improved through the use of positive or negative CI mode (GC–PCI–MS or GC–NCI–MS).

Both EI and NCI modes can be integrated as a multiple ionization mode of GC–MS approach and this possibility was successfully applied in the simultaneous analysis of opiates,

APs, and KET and metabolite in hair [137]. In the corresponding paper, after extraction with an organic solvent, the samples (25 mg) were subsequently cleaned up and preconcentrated by SPE (Bond Elut Certify) and then subjected to derivatization with HFBA. In comparison with a previous study that used the same protocol for hair sample preparation, but employing only EI ionization mode [135], the proposed method gave lower LOD and LOQ values by using NCI. The sensitivity of opiates and APs was improved from 8- to 60-fold. Particularly in the case of KET and norketamine (NKET), the increase in sensitivity was about 200 times (LOQ of 50 pg/mg for EI vs. LOQ of 0.25 pg/mg for NCI) and about 2,000 times (LOQ of 50 pg/mg for EI vs. LOQ of 0.025 pg/mg for NCI), respectively. Apart from the enhancement of sensitivity, this strategy offers several advantages, such as avoiding wrong results and misinterpretation of correct results, since the GC–NCI/MS can be used to confirm the results of GC–EI/MS. Hence, there is no doubt that integrating these two different ionization techniques for hair testing can provide valuable and valid information in the fields of forensic science and clinical toxicology.

More advanced techniques of detection such as MS/MS or HRMS also have been reported in combination with GC for the analysis of different drugs of abuse in hair samples.

MS/MS detection offers additional specificity with secondary fragmentations and can circumvent some problems, such as the issue related with co-elution. Analytical methods based on the combination of GC with QqQ and IT mass analysers have been employed for the analysis of a variety of drugs of abuse in hair samples [89, 138–141]. In this regard, a sensitive GC–CI-(IT)MS/MS method was developed for quantification of COC and three of its metabolites [141]. After acid hydrolysis, hair samples (50 mg) were extracted with an automated SPE (Isolute HCX). Using an IT mass spectrometer in positive CI mode, LOQs of 100 pg/mg were determined for anhydroecgonine methylester (AEME) and 50 pg/mg for EME, COE and parent COC. In order to achieve a better sensitivity, the possibility of injecting a sample volume higher than 1 mL (common used volume) was investigated in this work. A retention gap was installed prior to the chromatographic column, thereby enabling the injection of higher sample volumes (3 mL), as well as avoiding the introduction of matrix interferences into the GC

column. This approach proved to be sensitive and useful as a quantitative confirmatory test for COC and its metabolites found in hair. Nevertheless, BZE was not included in this method, which is a weak point since this compound is the main COC metabolite and an important marker of COC abuse.

In another study, a GC–EI-(IT) MS/MS method to detect cannabidiol (CBD), cannabinol (CBN) and THC in hair samples was established [89]. After alkaline digestion and subsequent acetylation, hair samples (10 mg) were subjected to a procedure based on HS-SPME. In comparison with another work that used the same protocol for the hair sample preparation, but with analysis using GC–EI/MS [93], the proposed method gave lower LOD and LOQ values for the three cannabinoids (e.g. for CBD, the increase in sensitivity was about ten times). However, the proposed approach appears unsuitable for the detection of THC-COOH, the main THC metabolite, which is extremely useful in cases when discrimination of the external contamination from cannabis consumption is required.

As mentioned in the previous section, the concentration level of THC-COOH in hair is lower than that of the parent drug THC because of the weak incorporation of the acidic metabolite into the hair matrix. The proposed cut-off concentration for the detection in hair of THC-COOH is 0.2 pg/mg in the case of the SoHT [19] and 0.05 pg/mg in the case of the SAMHSA [20]. Satisfying these analytical requirements in routine hair analysis has been possible by means of the GC–NCI-(QqQ)MS/MS method [139]. The authors used alkaline digestion of 25 mg hair aliquots, followed by LLE and, lastly, the hair extracts were subjected to HS-SPME after derivatization with pentafluoro-propionic anhydride/pentafluoro-propionic anhydride-pentafluoro-2-propanol (PFPA/PFPOH). Good analytical precision with RSDs 13.4% in real samples were obtained in both intra-day and day-to-day experiments. The LOD and LOQ of analytical method were determined to be 0.02 and 0.05 pg/mg, respectively. It thus appears a highly suitable analytical tool for the identification of THC-COOH at very low amounts.

Due to its high specificity and sensitivity, GC coupled with HRMS was used for the qualitative and quantitative analysis of AP and its major metabolite, MP, in human hair [142]. Hair samples (30 mg), after overnight acidic hydrolysis and subsequent derivatization with trifluoroacetic anhydride (TFAA), were subjected to GC–HRMS

selective ion monitoring (SIM). The HRMS experiments were performed using a three-sector, double focusing MS. The LODs were 9 pg/mg for MP and 21 pg/mg for AP. The GC–HRMS and low-resolution MS (LRMS) were compared with respect to their performance in the determination of these analytes. The LRMS experiments were performed with a single-quadrupole MS. The use of HRMS displayed several advantages for the determination of the two APs, such as better selectivity, better linearity, and lower LOD and LOQ values. In 19 out of the 30 hair samples collected, AP was not detected by the LRMS SIM technique. However, using the HRMS SIM method, it was successfully confirmed. In this regard, in cases of low concentration of AP in hair, the HRMS SIM technique appears useful for eliminating co-eluents, which are present in single-quadrupole LRMS because of matrix background or interference of cosmetic treatments.

The application of two-dimensional GC (2D GC) in the drug analysis field has also been reported in the literature, since this technique has advantages over the classic one-dimensional GC (1D GC), such as improved peak capacity, often enhanced sensitivity and the feature of "structured" chromatograms. In this regard, a procedure for the determination of THC-COOH in hair using 2D GC coupled to MS with NCI mode was described by Moore *et al.* [143]. In the paper, after alkaline digestion, the samples (20 mg), were subsequently cleaned up and preconcentrated by SPE (Bond Elut Certify) before being subjected to derivatization with TFAA and 1,1,1,3, 3,3-hexafluoro-2-2-propanol (HFIP). The results of this approach demonstrate detection of THC-COOH in hair using a single quadrupole mass detector at a concentration level of 0.05 pg/mg, which is the cut-off level currently proposed by the SAMHSA. Furthermore, the use of two serial GC columns allows minimizing the matrix background and, thus, the use of deuterated analogues as internal standard can be avoided. The low sensitivity achieved enables the avoidance of false-negative results regarding cannabis consumption.

2D GC has also been coupled to TOF mass analysers for profiling drugs of abuse in hair samples [144]. In the corresponding paper, after acidic incubation overnight, the samples (20 mg) were subsequently cleaned up and preconcentrated by SPE (Bond Elut Certify) before being subjected to derivatization with N-methyl-N-(tert-butyldimethyl) tri-fluoroacetamide (MTBSTFA), which efficiently reacted with a broad range

of multiclass analytes, including opiates, COC and metabolites, and BZDs. The method allows non-targeted analysis with the ability to detect multiple drugs of abuse at levels commonly found in users. Therefore, it may be useful for drug analysis in workplace, doping controls or reissuing driving licenses, in which a general profile of drug use is required or in cases in which drug use is suspected but unproven.

Gas chromatography-based methods for the determination of new psychoactive drugs in hair samples can also be found in the scientific literature. Procedures using GC–EI/MS for mephedrone [145], piperizines [146] and phenylalkyl-amine derivates [147] have been published, which have presented adequate precision and accuracy. The proposed methods can be useful for routine hair analysis, as these are sensitive and specific enough for detecting small amounts of these compounds using relatively small quantities of sample (20–50 mg) and a single quadrupole MS, which is an accessible tool in most laboratories nowadays.

*4.3. Capillary electrophoresis*

Besides LC and GC, capillary electrophoretic methods have also been successfully applied to determine both therapeutic and illicit drugs and their metabolites in hair samples, as can be seen in Table 3. With respect to the detection system, DAD detection is still currently employed despite the short internal diameter of the capillary that limits the concentration sensitivity. In addition, the coupling of capillary electrophoresis (CE) with a MS detector has been successfully applied to hair analysis. Of the different modes of CE, micellar electrokinetic chromatography (MEKC) and capillary zone electrophoresis (CZE) are the most widely used modes for drug testing in hair. In order to deal with the small amount of analytes injected in CE, various approaches have been developed, mostly using preconcentration procedures such as sweeping techniques or field-amplified sample injection (FASI).

A preconcentration strategy known as cation-selective exhaustive injection (CSEI) combined with sweeping and MEKC–DAD to determine MP, KET, MOR and COD in hair samples was reported by Lin *et al.* [148]. Using this strategy, the authors increased the sensitivity of the analytes under study up to 1,000-fold compared to MEKC–DAD with a conventional hydrodynamic injection. Using 10 mg aliquots of hair, LOD values ranged from 50 pg/mg for MP and KET to 200

**Table 3.** Capillary electrophoretic/electrokinetic-based methods for the determination of drugs of abuse in hair samples.

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| AP, MP, MDA | CSEI-Sweeping-MEKC-DAD | Washing with an aqueous solution of 5% v/v detergent; Extraction with 1 M NaOH (30 min, 70ºC); LLE with cyclohexane | 250 | 20 | [214] |
| MP, COD, MOR, KET | CSEI-Sweeping-MEKC-DAD | Washing with 1% SDS, water, MeOH; Extraction with 0.1 M HCl (2 h, room temperature); LLE with EtAc | 50-200 | 10 | [148] |
| 6-AM, MOR, AP, MP, MDA, MDMA, COC, BZE, ephedrine | FASI-CZE-ESI-IT-MS | Washing with aqueous solution 0.3% Tween-20; Extraction with 0.1 M HCl (overnight, 45ºC); LLE with Toxi-Tubes A | 5-50 | 100 | [149] |
| AP, MP, MDA, 6-AM, MDMA, EP, COC, MOR, COD, BZE | FASI-CZE-ESI-TOF-MS | Washing with aqueous solution 0.3% Tween-20; Extraction with 0.1 M HCl (overnight, 40ºC); LLE with Toxi-Tubes A | 6-100 | 100 | [150] |
| Abused drugs | FASI-CZE-ESI-TOF-MS | Washing with aqueous solution 0.3% Tween-20; Extraction with 0.1 M HCl (overnight, 45ºC); LLE with Toxi-Tubes A | – | 100 | [151] |

pg/mg for MOR, which are in accordance with the cut-offs recommended by the SoHT [19]. With good sensitivity and good reproducibility (RSDs <11% for all the analytes under study), this method proved to be useful for confirming the presence of trace amounts of these illicit drugs in the hair of the addicts.

The couplings of CE with MS for drug analysis in hair samples have also been reported in the literature, since this hyphenation combines the high efficiency of CE with the universality, selectivity and sensitivity of MS. Many efforts have been carried out for the developments of interfaces between CE and MS, being the ESI the most common interface. Gottardo *et al.* [149] developed a hair analysis method for the simultaneous determination of different drugs of abuse, including opiates, APs, COC, BZE and ephedrine, by means of CZE with ESI-(IT)MS detection. In

this paper, the samples (100 mg) after acidic incubation overnight, were subsequently cleaned up by LLE and then injected under FASI stacking injection, achieving LODs (between 5 and 50 pg/mg) below the cut-off values proposed by the SoHT [19] for detecting these analytes in hair samples, except for BZE (LOD 100 pg/mg). This could represent a problem because BZE is known to be incorporated into hair to a lesser extent than its parent drug. Analytical precision was fairly acceptable with RSDs 3% for migration times and 22% for areas in real samples, in both intra-day and day-to-day experiments. Quantification also proved acceptable, even without the use of deuterated internal standard (nalorphine), thus offering a cheaper alternative to GC–MS for quantitative analysis. For peak identification, the IT mass spectrometer was set to perform $MS^2$ on the molecular ion of the selected compounds. Thus, there may be applications for the presented method in toxicology laboratories for rapid, sensitive and unequivocal confirmation of the studied analytes, except BZE.

More recently, analyses of hair samples have also been carried out by means of CZE with ESI–TOF–MS detection, which offer the advantage of accurate mass measurements [150–152]. For example, Gottardo *et al.* [150] developed a CZE–ESI–TOF–MS method for the analysis of opiates, APs, COC, BZE and ephedrine in hair samples, using an LLE procedure and injection under FASI conditions for the sample clean-up and preconcentration of the analytes, providing LODs for the different drugs in the range of 6–25 pg/mg, except for BZE (LOD 100 pg/mg). Analytical precision in real matrices was typically characterized by CVs 24% in both intra-day and day-to-day experiments. Drug quantification was tested by using a single non-deuterated internal standard (folcodine). This method provides adequate sensitivity to allow the determination of all the drugs tested in concentrations lower than reported in the SoHT guidelines [19], except in the case of BZE, while it provides moderate accuracy. This method can be regarded as a valuable and reliable tool for toxicological analysis of hair in therapeutic drug monitoring, drug rehabilitation program, doping control, etc. since it provides high identification power.

Later, the same group reported some preliminary results obtained using an analogue method to screen a wide range of drugs, both therapeutic and illicit, and their metabolites in hair samples [151]. This work shows the potential of CZE–ESI–TOF–MS for psychoactive drug analysis in hair,

which could become a very powerful tool for broad-spectrum monitoring of subjects under- going a drug rehabilitation program, with the purpose to determine abstinence and compliance to the therapy. Unfortunately, the proposed method did not result suitable for the BZDs determination, possibly due to poor extraction from the hair matrix by acidic digestion.

Gottardo *et al.* [152] also investigated the possibility of using non-volatile buffers traditionally used in CE–DAD also in CE–MS for the analysis of drugs of forensic interest in hair. The use of a phosphate buffer proved to be suitable for CZE–TOF–MS analysis, thus increasing the versatility of the CE–MS methods, in comparison to LC–MS methods. The LODs of the different tested drugs were found to range from 2 pg/mg for MOR and COD to 10 pg/mg for COC, MDA and MDMA, which proved to be slightly better than those obtained when the same authors used ammonium formate buffer under the same conditions [150].

Typically, the CZE–MS methods for drug analysis in hair require relatively large amount of sample (100 mg) in order to achieve good sensitivity, which could be a problem when sample availability is limited.

In summary, it should be highlighted that the LODs reported in Table 3 for CE-based methodologies are low enough for determining drugs, both therapeutic and illicit, and their metabolites in hair samples. However, it is important to note that all of the papers published in recent years in which CE is the separation technique used are based on the use of LLE in the clean-up and preconcentration step. This may be considered as a drawback due to the great amount of organic solvents used. In line with the trend towards environmentally friendly procedures, it would be of great interest to test the performance of greener strategies, such as combinations between CE and microextraction techniques like in-line SPE–CE and single drop microextraction (SDME)-CE.

### *4.4. Novel approaches*

In recent years, several methods have been proposed based on matrix-assisted laser desorption/ionization MS (MALDI–MS) and Direct Analysis in Real Time-MS (DART-MS) for the accurate and specific determination of xenobiotics of toxicological and forensic interest in hair, which are summarized in Table 4. Vogliardi *et al.* [153, 154] have described a fast screening method for the detection of COC in hair by

**Table 4.** MALDI and DART-based methods for the determination of drugs of abuse in hair samples.

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (pg/mg)* | *mg of hair used* | *Ref.* |
|---|---|---|---|---|---|
| COC | MALDI-MS | Washing with 10% SDS, water, acetone; Pulverization and extraction with ACN/TFA (1:1), (5 min) | LOD: 100 | 2.5 | [153] |
| COC, BZE, COE | MALDI-MS | Washing with water, acetone, hexane; Incubation in MeOH/ TFA (15 min, 45°C) | – | 1 | [154] |
| MP | MALDI-TOF MALDI-FTICR | Washing with 0.5% SDS, water, MeOH | – | Single hair | [155] |
| MP | MALDI TOF | Washing with 0.5% SDS, water, MeOH | – | Single hair | [156] |
| COC | Imaging MALDI-MS | Washing with water, acetone, hexane | – | Single hair | [157] |
| THC | DART-MS | Washing with DCM (3 mL, 3 min, twice) | – | Single hair | [160] |

MALDI–TOF. After extraction of hair samples by incubation in methanol (MeOH)/trifluoro-acetic acid (TFA), a sensitive analysis was achieved by using a MALDI spot preparation based on a multilayer approach. The spot preparation consisted of a graphite layer, the sample (0.2 mg of powdered hair samples) and electrosprayed α-cyano-4-hydroxycinnamic acid (CHCA) matrix on the top. With this sample preparation, a LOD of 100 pg/mg could be reached for COC. However, in the same study, the direct MALDI-TOF analysis on a single intact hair (4-cm segment) containing 100,000 pg/mg of COC was also investigated, but no valid results were obtained.

MALDI–TOF and MALDI–Fourier transform ion cyclotron resonance (FTICR) MS approaches have also been described, in this case, for the detection of MP in human hair samples [155, 156]. A longitudinal section of a lengthwise manually-cut single human hair shaft from a chronic MP user showed a barcode-like image (generated with repeated intakes of MP by monitoring an MP- specific product ion) which was confirmed by ultra-high resolution mass spectrometry by FTICR. This approach appears to be very promising, once the hair sample preparation can be automated.

Sometimes, multiple molecules with the same m/z value are detected. In such cases, imaging MS is effective.

Using this technique, each ion derived can be separated from its specific fragment ions. In this way, Porta *et al.* [157] used a triple quadrupole linear ion trap to perform MALDI imaging MS experiments on single hairs (6-cm segment) of chronic COC users and were able to detect COC concentrations as low as 5,000 pg/mg. By longitudinal scanning of a single hair, the authors could obtain detailed historical information concerning drug intake over several months. The analyses were accomplished by spraying CHCA, 4-chloro-a-cyano-cinnamic acid, or (E)-2-cyano-3-(naphthalen-2-yl) acrylic acid as MALDI matrices. Compared to traditional methods based on LC–MS/MS or GC–MS(/MS), which require to segment the hair to obtain spatial resolution, MALDI–MS imaging, with a straightforward sample preparation beforehand, allowed a spatial resolution of 1 mm to be obtained and, thus, the chronological information about COC consumption contained in a single intact hair over several months could be monitored. In addition, the very high longitudinal spatial resolution of this approach (1 mm) could potentially provide an estimate of the time of drug intake in days, versus a month when using conventional segmented hair analysis methods.

Although these results represent major scientific achievements, the analysis of single hairs can easily lead to misinterpretation. The growth cycles of individual hairs are not synchronous: 80–90% is in the anagen phase and growing, while the remainder of the hairs is in the regressing catagen or resting telogen phase and not growing [7, 61].

Hence, single hair analysis can cause false-negatives and incorrect conclusions with regard to retrospective timeline assessment, due to these differences in growth cycles. This has been observed in practice by Musshoff *et al.* [158] while analysing four single hairs of a drug abuser with MALDI imaging MS. Using a sophisticated Orbitrap MS instrument, they were able to detect COC, its metabolites and cannabinoids down to sub-ng/mg levels in two single hairs, but the other two hairs did not show any drugs at all. Averaging the results of several hairs from one individual monitored by MALDI hair scanning may overcome this problem but, to the best of our knowledge, this has not been demonstrated.

As an alternative, an ambient ionization MS hair scan approach was proposed targeting entire locks of hair without the addition of MALDI matrix or any other sample preparation by means of Direct

Analysis in Real Time (DART)-MS. DART was introduced in 2005 by Cody *et al.* [159] as a new ionization technique for fast and direct analysis under ambient conditions. A glow discharge inside the DART source is used to yield metastable species of a heated gas (e.g. helium), which desorb analyte ions from a sample surface at elevated temperatures. Duvivier *et al.* [160] developed a novel DART-Orbitrap MS hair scan method using THC as a model compound. Instead of using digestion of hair or digestion of multiple one-three centimetres hair segments followed by conventional LC/or GC/MS analysis, complete locks of hair were tested under ambient conditions using DART. By using entire locks of hair, the analysis is not hampered by differences in growth cycles between individual hairs. The preliminary results obtained showed that Orbitrap MS systems are capable of pushing the DART hair scan LOD into the sub-ng/mg range, which is highly relevant for single dose drug administrations and retrospective timeline assessments, such as in drug-facilitated crime cases. The sensitivity may be increased towards the pg/mg range using a QqQ mass spectrometer instrument.

In summary, it can be noted that both MALDI and DART methods could be ideally suited when fast screening of a large number of samples is required. However, at the present, the high cost of these instruments limits their widespread use.

## 5. Interpretation of results

As discussed, hair allows drug exposure to be assessed and documented in a wide range of situations, but extreme caution is needed in the interpretation of the results obtained before a report is issued. Detection of a drug is not sufficient to identify drug use because several factors may have an influence on drug disposition in hair, which are discussed in detail below.

### *5.1. External contamination*

The most important drawback of hair analysis is the possibility of providing false-positives results, particularly in those situations where the individual is exposed to the drugs, but does not actively consume them [11, 23, 29, 32, 33, 45, 161, 162]. Environmental contamination of a drug onto the exterior of the hair is common in users of illicit drugs that can be smoked such as marijuana, COC, AP, MP and heroin. Moreover, handling the drug powder and rubbing hair with the hands is another source of passive

contamination [32, 163–166]. If adequate measures are not taken, the risk of reporting false-positive results increases, which is unacceptable, especially if there are legal implications of drug consumption [9]. Currently, laboratories use two complementary procedures to minimize this effect. The first is decontamination of hair samples by washing them before analysis and the second is related to detection of drug metabolites.

Several decontamination procedures are described in the literature [5–7, 23, 24, 29, 30, 40], but there is no agreement on which procedure must be used [6, 9]. Indeed, it is assumed that the elimination of the totality of the externally deposited drug is not achieved even after laborious washing procedures [6, 9, 32, 33, 40, 163, 167–169]. Schaffer *et al.* [170] compared three different washing procedures for the differentiation of COC from external contamination versus ingestion in hair samples exposed to COC at 1 mg mL 1 for 1 h. They compared three washing methods: (1) MeOH, (2) 2-propanol/phosphate buffer (three buffer washes) and (3) 2-propanol/phosphate buffer (five buffer washes). The results obtained showed that even after extensive washing,100% removal of external contamination was not possible. However, it is important to note that the method used by the authors to contaminate the hair, involved soaking it in COC solution, an event unlikely to occur. Therefore, the 2-propanol/ phosphate buffer (three buffer washes) would likely be appropriate for a less severe contamination such as from smoke or powder. However, decontamination procedures are not generally sufficient for adequate differentiation between drug use and environmental contamination.

For this reason, criteria have been proposed consisting of establishment of concentration ratios between the last wash (W) and the hair sample (H). After several washes, measurement of a drug in the solution is compared with the measurement of the drug in the hair. In general, when drugs are detected in hair and not in the wash residue, drug use is indicated, whereas, when the levels detected in the wash residue are greater than the levels in the hair, it is likely an indication of passive contamination [171]. Tsanaclis and Wicks [171] reported on the results from the analysis of 216 hair samples and the corresponding methanolic wash fractions. The samples were obtained from a population where external contamination could be expected (drug related police investigation cases). The analyses were used to determine the wash-to-

hair (W-H) ratios. The authors reported that a W-H ratio less than 0.1 or null it would tend to indicate drug use; a W-H ratio above 0.1 but less than 0.5, is likely to indicate possible drug use with a degree of external contamination; and finally, a W-H ratio greater than 0.5 is likely to indicate that the source of most of the drug in the wash residue is from external contamination. From the data, between 74% and 100% of the analytes studied produced W-H ratios less than 0.5, in particular in cannabis (93%) and COC (95%), where external contamination is more likely because of the way the drug is used. Meanwhile, 97 of the 891 positive test results had W-H ratios greater than 0.5, demonstrating that approximately 11% of all positive tests were consistent with a high probability of contamination and were inconclusive concerning drug use.

The second procedure to minimize false-positive results is the detection of drug metabolites, specifically those that derive solely from endogenous metabolism [11, 29, 172, 173]. In this regard, the SoHT and SAMHSA organizations established cut-off levels for the most commonly abused drugs and their metabolites. For instance, a positive sample for COC should include: COC 500 pg/mg and at least one COC metabolite at a concentration 50 pg/mg [19, 20]. Villain *et al.* [174] examined heroin markers in the hair of narcotic police officers to determine active or passive exposure. This is an interesting case where a police officer and a clerk were arrested for drug trafficking or reselling seized drugs. The hair samples were decontaminated by washing them twice with dichloromethane (DCM), incubated overnight in 0.1 N HCl, cleaned up by means of LLE and, finally, analysed by GC–MS. The police officer and the clerk who were allegedly selling the drugs tested positive for 6-AM and MOR (cut-off opiates level: 200 pg/mg). The police officer tested positive in chest hair (6-AM: 500 pg/mg, MOR: 200 pg/mg) while the clerk resulted positive in head hair (6-AM: 800 pg/mg, MOR: 400 pg/mg). They both claimed external contamination. Other officers in the same workplace area were tested using either chest or head hair and, in all eleven cases, there was no heroin or no other drugs detected.

In terms of THC external contamination, the detection of the carboxy metabolite (THC-COOH) is widely used to prove active cannabis consumption [12, 175, 176]. For example, Uhl and Sachs [177] reported a case in which hair samples from a couple living together in an apartment tested positive for THC and CBN. The male subject admitted

smoking marijuana whereas the female subject denied any consumption. Analysis of the hair samples for THC-COOH showed a high level (>6.6 pg/mg) in the sample of the male and negative results (LOQ 0.1 pg/mg) in the sample of the female. Since THC-COOH is generally present in the low pg/mg range in the hair of cannabis users and sometimes not detectable at all despite using extremely sensitive analytical methods or after confirmed consumption [175, 178], the missing detection of this oxidative metabolite does not prove external contamination. A specific marker for an external contamination of hair could give useful additional information. $\Delta^9$-Tetrahydrocannabi-nolic acid A (THCA-A) seems to be a promising candidate for this purpose. This compound is the non-psychoactive precursor of THC and the main cannabinoid in fresh cannabis plant. The incorporation of THCA-A into hair has been investigated in a pilot study which revealed that this compound does not get incorporated into hair after oral intake of relatively high doses of THCA-A for 30 days [35, 165]. Thus, this led to the assumption that THCA-A found in hair may arise from external contamination caused by, for example, sidestream marijuana smoke or contaminated hands after being in contact with cannabis plant [179]. This is a different twist on external contamination, looking for the contaminant as opposed to trying to clean out the contaminant.

It should be noted, however, that conversion of some drugs to their metabolites may occur as an artefact during sample preparation, such as the hydrolysis of COC to its metabolite BZE, or heroin and 6-AM conversion to MOR [40, 135, 180, 181]. For example, Wu *et al.* [135] investigated different digestion/extrac tion procedures by analysing MOR and 6-AM in authentic hair samples. Under basic digestion, 6-AM was totally hydrolyzed to MOR. As such, the SAMHSA recommends also the use of metabolite to parent drug ratios to report positive results, e.g. BZE to COC ratio higher than 0.05 for COC ingestion, and 6-AM to MOR ratio higher than 1.3 in the case of heroin consumption [20]

Stout *et al.* [32] studied in vitro COC contamination in hair donated by five subjects. To do so, hydrochloric COC powder was externally applied to hair samples and the concentrations of COC analytes determined over a ten-week period during which the hair was subjected to regular shampooing. Three commercial analytical laboratories analysed the hair samples under three protocols: no decontamination procedure, individual

laboratory decontamination and decontamination by an extended buffer procedure. For the non-contaminated hair, the BZE to COC ratio was more significant. Moreover, the metabolite to parent ratios of BZE to COC increased over the ten-week period. From 21 days post-contamination to the end of the study, the BZE to COC ratios was greater than 0.05, the proposed Federal Mandatory Guidelines requirement (SAMHSA). The suggestion to use this ratio as a criterion was shown not to be effective, at least in this study. Obviously, this fact does not take away the importance of hair analysis in the field of toxicology. On the contrary, it highlights the extreme care that should be taken in the interpretation of results.

There are some additional attempts to reduce the number of false-negatives and, along these lines López-Guarnido *et al.* [161] reported a method based on GC–MS to assess COC use. Their proposal was to combine the usual cut-off recommended by the SoHT and the ratio of BZE:COC (>0.05) with the ratio COE:COC (>0.02).

### *5.2. Influence of cosmetic treatment*

An issue regarding hair analysis that should not be overlooked is the possible effect of various hair products and chemical treatments (e.g. bleaching, colouring, perming) on the measurement of drugs of abuse in hair [23, 26, 30, 182]. In a study conducted on the influence of bleaching on stability of AP-type stimulants in hair, results showed that the concentrations of all drugs decreased in bleached hair in comparison with non-treated hair [183]. Likewise, this type of treatment affects the stability and decreases hair concentrations of opiates, COC and BZDs [11, 30]. That said, low abuse of drugs may not be detected in hair treated with bleaches, perms or dyes, but regular drug abuse will likely be detected even after chemical treatment of hair, although the concentrations of drugs may be reduced. Conversely, Skopp *et al.* [184] and Hill *et al.* [167] reported that cosmetic hair treatments may also damage hair by altering its porosity and increasing its accessibility to external contamination. Hence, the more porous the hair the more drug uptake will occur from the environmental contamination [166]. Porosity can also be caused by racial differences or bad hair preservation [167]. Shampooing of hair has little effect on removing drugs already incorporated in hair. In contrast, shampooing may be effective at removing drugs absorbed in hair due

to environmental contamination.

Nowadays, most shampoo products made with cannabis products contain less than 1% THC, but some of the products may contain between 1% and 3%. Analysis of the commercially available shampoo Cannabio showed the presence of 412 ng/mL of THC, 4,079 ng/mL of CBD and 380 ng/mL of CBN. To explore the possibility of false-positives laboratory results due to the use of these shampoos, three subjects washed their hair with Cannabio shampoo once daily for two weeks [30]. After this time, the hair samples were analysed for THC, CBD and CBN, and were found to be negative for the presence of these compounds. The LODs for THC, CBD and CBN were 50, 20 and 10 pg/mg, respectively. To study the effect of longer exposure time to Cannabio shampoo, the same authors incubated hair samples with 10 mL water/Cannabio shampoo (20:1, v/v) for 30 min, 2 and 5 h. After an incubation period of 30 min, the analysis of hair by GC–MS did not reveal any presence of cannabinoids. When samples were incubated for 2 and 5 h, the samples tested positive for CBD and CBN. Thus, the use of Cannabio shampoo can cause drug free hair to test positive for CBD and CBN but not for the primary psychoactive drug THC.

In summary, it is very important always to consider the cosmetic history of a hair sample in the interpretation of hair analysis results.

*5.3. Influence of hair colour*

Natural hair colour is another important feature to be kept in mind in the evaluation of drug concentration in hair. In this respect, it has been shown that abused drugs bind to melanin, both eumelanin (the most common type of melanin produced in black and brown hairs) and pheomelanin (responsible for red hairs) [6, 23, 30, 31, 185, 186]. The darker the hair, the greater the melanin content, as this is what constitutes or makes up the pigment. Thus, the incorporation and binding of drugs in the hair is much greater in pigmented versus non-pigmented hair. As a result, there are higher concentrations of drugs and their metabolites incorporated into dark versus light hair, when given the same dose of the drug [5, 9, 24, 187, 188]. This bias towards increased drug concentrations in darker hair could theoretically lead to a difference between positive or negative hair drug test outcomes. As there is a hair colour bias and many different cultures and races tend to have a specific or common hair colour, this does imply a potential racial bias to

hair testing, which can be defined as hair colour.

For example, hair colour bias has been reported for COD in human hair, supported by a study in which the authors administered 30 mg of oral COD syrup three times a day for five days to a Caucasian and a non-Caucasian (Asians) group of subjects [186]. Plasma samples were collected for 24 h and hair samples were collected on weeks four, five, six, and seven. There was a correlation between the COD concentration and the total melanin content, with the $r^2$ equalling 0.73. For both Caucasian and Asian black hair, the mean COD concentration was 1,429 pg/mg. For brown hair, blond hair and red hair, the mean COD concentrations were 208, 99 and 69 pg/mg, respectively. In Asian black hair (N=12), the mean concentration was 2,564 pg/mg, compared to Caucasian black hair at 865 pg/mg. Using the SAMHSA guideline of 200 pg/mg as the cut-off for COD in hair testing protocol, 100% of subjects with black hair and 50% subjects with brown hair in their study would have been reported as positive. In contrast, subjects with blond or red hair would have tested negative. Not only were there differences in terms of hair colour but also a difference in the type of hair based on the ethnicity of the donor was observed. Similar results were obtained by Ropero-Miller *et al.* [33] with COC concentrations significantly higher in dark hair types, including African–American individuals. The authors suggested that the use of BZE to COC ratios and extensive decontamination wash criteria greatly reduce positive hair in vitro testing results in contaminated hair.

Borges *et al.* [189] studied the incorporation of AP, which is a basic compound and N-acetylamphetamine (N-AcAP), an analogue of AP which is non-basic, to evaluate the role of basicity in hair colour bias. Does some sort of cationic binding occur? For this model, male Long-Evans rats, both black pigmented and white non-pigmented, were dosed with AP or N-AcAP and their hair evaluated for drug content. Black hair from rats dosed with AP (N=8) was found to contain 6.44 ng/mg. White hair from the same rats contained 2.04 ng/mg. In contrast, no difference in N-AcAP content was found between black hair (0.87 ng/mg) and white hair (0.83 ng/mg) from rats dosed with N-AcAP (N=8). The results from this study clearly demonstrate that drug basicity can lead to a hair colour bias, whereas a lack of basicity appears to eliminate hair colour bias. Thus, the pKa or the basicity of a particular compound plays a role in the drug's binding capacity to hair.

In this regard, basic drugs bind with high avidity to eumelanin, which is in higher concentration in dark hair. These research findings give credence to the concept of hair colour bias for some drugs. However, preliminary studies indicate that cannabinoids concentrations are similar in dark and light hair [175].

Kronstrand *et al.* [190] conducted COD controlled dosing cases. Nine individuals were given a single oral dose, plasma was collected over 24 h, and then hair samples were collected once a week for one month. The authors found a correlation between COD concentration and the melanin. The melanin increased as COD increased, with an $r^2$ of 0.86. Eumelanin content was also correlated with COD at an $r^2$ of 0.90. In an attempt to eliminate bias, the authors suggested that drug concentrations in hair could be normalized for hair colour by expressing this as a function of the melanin concentrations, thereby reducing the influence of a person's hair colour on the interpretation of hair test results.

In light of the findings discussed above, the following question arises: does removal of the melanin from hair extracts prior to hair analysis may compensate for the hair colour bias due to melanin? In one study, the effect of melanin removal by centrifugation of hair digests on COC concentrations was investigated [191]. The authors digested hair samples from five COC users with proteinase K. After centrifugation at 2,000 rpm, the supernatant and the melanin pellet obtained were analysed separately. A mean of 8.8% of the total COC concentration (supernatant and pellet) was left behind in the pellet. The same experiment was repeated, except that the melanin pellet was re-digested with 0.1 N HCl. After re-digestion of the melanin pellet, the mean COC concentration in the pellet was 3.8% of the total COC concentration in hair. Thus, removal of melanin from hair digests by centrifugation does not eliminate hair colour bias when interpreting COC concentrations.

Furthermore, it is possible that genetic differences play an important role in melanin concentrations and in hair porosity, and as such, influence drug incorporation [192].

### *5.4. Dose-concentration relationship*

One of the major questions asked is whether the results of the quantitative hair analysis can indicate the amount of drug used. Studies have been conducted on this matter, some of them showing a correlation between dose and hair concentrations [28, 193,

194], but this relationship is generally weak.

Ropero-Miller *et al.* [194] demonstrated dose-related concentrations of COC analytes in human hair after controlled subcutaneous COC administration. The study was performed with eight volunteers enrolled in a ten-week inpatient clinical study. During the low-dose week, they received 75 mg/70 kg on alternatives days whereas, for the high doses, they received 150 mg/70 kg. The authors observed considerably inter-subject variability in the maximum concentrations for low doses, ranging from 1.7 to 15 ng/mg, and for high doses, ranging from 5.1 to 27 ng/mg. However, in the same person, the mean peak concentration in hair after low dosing was around half the concentration observed after high-dose administration. In a similarly designed experiment performed with ten volunteers residing in a secure research ward, Scheidweiler *et al.* [28] observed a significant dose-concentration relationship in the hair samples. Maximum hair concentrations were found one to three weeks after low and high doses. Considerable inter-subject variability was observed in hair concentrations. However, dose-concentration relationships were consistent in nine out of ten subjects.

Kintz *et al.* [195] demonstrated that there was no correlation between the doses of administered heroin and the concentration of total opiates measured in hair samples through a controlled study in which subjects were exposed to heroin. Subjects were administered two or three doses of heroin hydrochloride intravenously on a daily basis. Heroin doses ranged from 30 to 800 mg per day. All special features of hair such as colouring, bleaches, etc. were noted. Concentrations ranged from 0 to 4.53, 0.38 to 10.11 and 0.71 to 5.20 ng/mg for heroin, 6-AM and MOR, respectively. No correlation between the doses of administered heroin and the concentrations of total opiates in hair was observed ($r=0.346$). However, when considering a single analyte, it was observed that the correlation coefficient seemed to be linked to its plasma half-life. A weak correlation coefficient corresponds to a drug with a short plasma half-life, and the correlation coefficient increases when plasma half-life increases, with $r=0.12$, 0.25 and 0.64 for heroin, 6-AM and MOR, respectively. These results suggest that using quantitative drug measurements in hair to determine the amount of drug ingested will remain inapplicable until more is known about the factors that may

influence the incorporation of drugs into hair and a way to reduce the observed variability.

The purity of the drug, the uncertain mechanism of incorporation of drug into hair, the metabolism of a drug, the frequency of abuse, and hair colour and cosmetic treatments play an important role in determining the amount of drugs and metabolites incorporated into hair [11]. As a consequence, it is not possible to determine the exact amount of drug that was used during the previous period [1, 11]. Some laboratories have proposed a classification for drug concentrations (low, medium and high concentrations) as a general guide to indicate the level of drug use, e.g. low concentrations correspond to a low or occasional use (e.g. once or twice per week). For example, the low, medium and high ranges of MP and AP were determined as 0.5–4.2, 4.2–24.5 and 24.5–608.9, and 0.1–0.4, 0.4–1.7 and 1.7–41.4 ng/mg, respectively, based on statistical data (N=2,070) [15]. Therefore, quantitative hair analysis gives an estimate of the intake of drugs over time [28].

In view of all findings discussed in this section, interpretation of hair results is a complex issue. Although a unique and uniform criterion has not been reported to date, different international agencies have provided a number of recommendations for a better understanding of hair testing results.

Lastly, it is important to highlight the possibility of positive results deriving from the ingestion of legal drugs. For example, AP and MP can be detected in biological specimens after consumption of medicines such as fenproporex and selegiline. 1-(3-Chlorophenyl) piperizine, found in seized MDMA pills, is a known metabolite of the antidepressant drug trazodone (sold under the name Desyrel) and nefazodone (sold under the name Serzone) [11].

## 6. Conclusions and future perspective

In recent years, hair has become a fundamental biological specimen and an alternative to the usual samples blood and urine for drug testing. However, hair is a very complex matrix and, in addition, drug concentrations are often small, so there is a need for the development of selective and sensitive methods of analysis. This is mandatory in many toxicological areas, such as doping control, therapeutic drug monitoring, drug-related deaths and driving license renewal, among others. In this respect, recent improvements in mass spectrometry instrumentation allow an accurate identification of the drugs, which is very helpful when

legal implications are involved.

An important drawback is the presence of different sources of possible bias in interpreting hair analysis results, which are sometimes difficult to handle. One of these is the external contamination of hair which can lead to false-positive results and, as such, further research is needed and should focus on the development of greener and more effective strategies for hair decontamination prior to analysis. Moreover, more efforts should be made to gain a wider knowledge about the mechanisms of drug incorporation into hair and how this is affected by factors such as melanin content, age and gender, among others, in order to be able to interpret the results in a more reliable way. In addition, the availability of multidrug sensitive analytical methodologies can help towards obtaining more accurate and detailed information about the analysed samples, thus avoiding misinterpretation of the results.

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### 1.2.2. URINE DRUG TESTING

Urine testing is one of the most common methods employed to detect (ab)use of DOAs. Over recent years, it has gained popularity due to the various advantages listed in Table 1. As is well known, this testing approach detects recent or new drug use, typically within 1-3 days after drug use [12, 13]. However, the detection windows depend on the drug type ingested (Table 3) and may be affected by several factors, such as frequency of use, amount taken, drug distribution kinetics, volume of fluid ingested prior collection, kidney function, liver function and lipid solubility, among others [36].

**Table 3.** Detection windows for various DOAs [13].

| *Substance* | *Urine* |
|---|---|
| Amphetamines | 2-4 days |
| Methamphetamine | 2-5 days |
| Barbiturates | Up to 7 days |
| Benzodiazepines | Up to 7 days |
| Cannabis (marijuana) | 1-30 days |
| Cocaine | 1-3 days |
| Codeine (opiate) | 2-4 days |
| Morphine (opiate) | 2-5 days |
| Heroin (opiate) | 2-3 days |
| Phencyclidine | 5-6 days |

Determination of the presence or absence of DOAs in urine is routinely carried out through a common workflow, which is depicted in Figure 8. As can be seen from the figure, urine drug testing generally involves an initial screening test, typically performed by immunoassay. Immunoassay screening tests are based on the use of a specific antibody-antigen binding system. This antibody-antigen binding interaction may reveal the presence of DOAs and their metabolites in biological matrices. Nowadays, immunoassays are well-established procedures for providing a quick and effective means to screen urine specimens presumptively [37]. A range of immunoassay-based screening methods are available and detailed information on these can be found in the literature [12, 38].

Another part of the screening process is urine validity testing to determine if the specimen has been tampered with in any way. To do so, the specimen is usually tested for creatinine, specific gravity, pH and oxidants (nitrites). When urine validity testing falls outside of the specified ranges of what is considered normal, the sample

submitted can be labelled as diluted, substituted, adulterated or invalid and should not be used for drug testing. Various criteria can be followed to discriminate, which are discussed elsewhere [13, 39, 40]. A short summary of these is included later in this section.

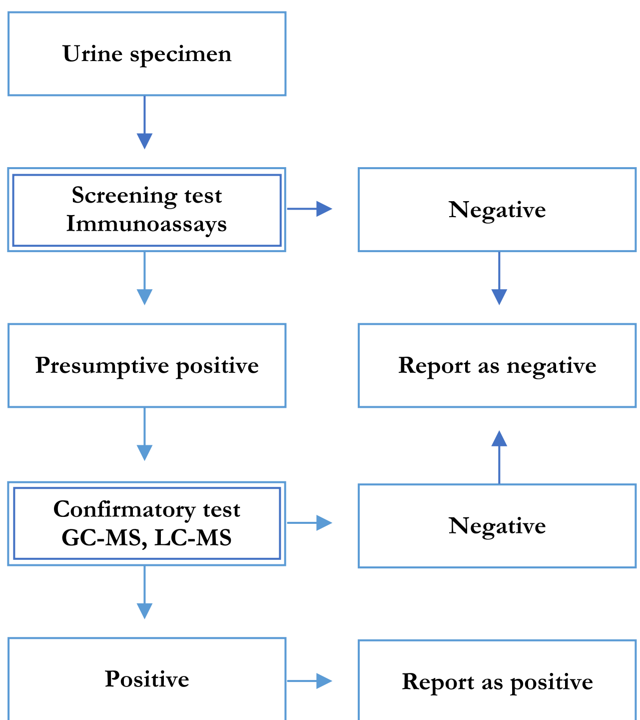


**Figure 8.** A typical workflow in urine drug testing for medical, legal and clinical purposes [37].

To distinguish between positive and negative results obtained from immunoassay screening tests, organizations such as the European Workplace Drug Testing Society and Substance Abuse and Mental Health Services Administration (SAMHSA) have established cut-off levels for the most commonly used DOAs and their metabolites [39, 41], which are listed in Table 4. Any value greater than or equal to the cut-off would be considered a presumptive positive result.

In the case of a positive screening test, a subsequent confirmatory test is performed by MS, coupled either to GC or LC, although electrophoretic methods have also developed into a significant field of research for detection of DOAs in urine in recent years. Cut-off concentrations for confirmatory testing have also been approved (Table

4). These cut-off values are lower than the screening limits and analytical methods must be sensitive enough to reach these levels to provide reliable results.

**Table 4.** Initial and confirmatory test cut-off concentrations for DOAs [39, 41].

| *Initial Test Analyte* | *Initial Test Cut-off (ng/mL)* | *Confirmatory Test Analytes* | *Confirmatory Test Cut-off (ng/mL)* |
|---|---|---|---|
| Marijuana (THCA) | 50 | THCA | 15 |
| Benzoylecgonine | 150 | Benzoylecgonine | 100 |
| Codeine/ | 300 | Codeine | 300 |
| morphine | | Morphine | 300 |
| Hydrocodone/ | 300 | Hydrocodone | 100 |
| hydromorphone | | Hydromorphone | 100 |
| Oxycodone/ | 100 | Oxycodone | 50 |
| oxymorphone | | Oxymorphone | 50 |
| 6-Acetylmorphine | 10 | 6-Acetylmorphine | 10 |
| Phencyclidine | 25 | Phencyclidine | 25 |
| Amphetamine/ | 500 | Amphetamine | 200 |
| methamphetamine | | Methamphetamine | 200 |
| MDMA/MDA/MDEA | 500 | MDMA | 200 |
| | | MDA | 200 |
| | | MDEA | 200 |

See Appendix I for the key to abbreviations.

Within this framework, this section focuses on the analytical methodologies employed for the determination of DOAs in urine samples. Analytical methods are discussed considering the sampling strategies, sample preparation, separation and detection, and, finally, interpretation of the analytical findings.

### Specimen collection and handling

Proper specimen collection procedures are critical for ensuring an adequate urine sample for drug testing. Nowadays, the Internet provides advice on a host of mechanisms for evading urine drug tests that range from simple to sophisticated strategies, as described later in this section. In such a context, direct observation is the most reliable method for specimen collection. However, this method is invasive and thus is not often practised. When specimens are taken without supervision, it is recommended that they are labelled as such so that the results are interpreted with caution. Urine samples that may have pertinence to a legal matter (e.g., those taken after a motor vehicle crash or as part of a court-ordered program) may require collection in a tamper-proof container, as well as needing a chain of custody. A less-

invasive collection method involves excluding coats and bags and using a specially prepared restroom without running water, soap or other chemicals. Although effective, only some laboratories can offer this service. In this case, it may sometimes help to have a staff member outside the door listening for running water. In addition, toilet water should be tinted, while the appearance and colour of the urine should be documented, and the temperature should be taken within four minutes of collection. Before any collection, the procedure should be explained to the user [13, 16, 39].

- **Sample preparation**

Sample preparation is a fundamental part of any analytical method and has a significant influence on most of the subsequent analytical steps and on data quality. Within this context, numerous sample preparation methodologies have been successfully developed for urine drug testing, always looking for a compromise between good recoveries for most of the analytes and cleanliness of the extract. The methods employed for sample preparation will be discussed for each determination technique considered in this section.

- **Analytical methodology**

In recent years, both chromatographic and electrophoretic techniques have been extensively exploited to identify and quantify different DOAs and their metabolites in urine samples. Within this context, a detailed discussion of the most relevant available analytical strategies for urine drug testing is given below.

*LIQUID CHROMATOGRAPHY*

The polar character of most DOAs makes them more suitable for analysis by LC than by GC since, in this case, a derivatization step prior to the analysis is not required. In this regard, LC now represents the analytical technique most widely used for toxicological analysis of urine samples, shown by the large number of applications developed. These applications, including the sample preparation, chromatographic separation and detection conditions, have been comprehensively reviewed in numerous recent documents, some of them are listed here [42-53]. With this in mind, based on these articles, a brief summary will be given here of the most relevant aspects of LC analysis for DOAs in urine, as well as a number of research works reported between 2009 and the present day that are worth mentioning {Table 5 [54-74]}.

**Table 5.** LC-based methods for the determination of DOAs in urine.

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| AP, MTD | *LC-UV* Stationary phase: $C_{18}$, 25 × 4.6 mm, 10 µm at 25ºC; Mobile phase: Isocratic; ACN/10 mM phosphate buffer (15:85, v/v); Flow rate: 1.0 mL/min | Magnetic SPE with graphene oxide particles; Injection into the system | 20 [b] (AP); 25 [b] (MTD) | [54] |
| EP, MP | *LC-UV* Stationary phase: $C_{18}$, 25 × 4.6 mm, 10 µm at 25ºC; Mobile phase: Isocratic; ACN/10 mM phosphate buffer (15:85, v/v); Flow rate: 1.5 mL/min | Magnetic SPE with carbon coated $Fe_3O_4$ particles; Injection into the system | 15 [b] (EP); 20 [b] (MP) | [55] |
| MP | *LC-UV* Stationary phase: InertSustain $C_{18}$, 100 × 4.6 mm, 5 µm at 35ºC; Mobile phase: Isocratic; ACN/30 mM ammonium acetate (10:90, v/v); Flow rate: 1.0 mL/min; Detection: 217 nm | DLLME with ionic liquids as extracting media; Injection into the system | 1.7 [b] | [56] |
| MOR, COD, OXCOD, OXMOR, HYCOD, HYMOR and their glucuronide metabolites | *HILIC-ESI-MS/MS (QTrap)* Stationary phase: Xbridge™ Amide HILIC, 150 × 4.6 mm, 3.5 µm at 30ºC; Mobile phase: Gradient; (A) 0.125% FA in 50:50 v/v ACN:water with 10 mM ammonium formate, (B) 0.125% FA in 90:10 v/v ACN:water with 10 mM ammonium formate; Flow rate: 1.4 mL/min | 1,000 µL urine; Off-line SPE with Oasis MCX; Evaporation/reconstitution | LOQ: 0.16-0.31 [b] | [57] |
| MOR, COD, M3G, M6G, C6G | *HILIC-ESI-HRMS (TOF)* Stationary phase: Zorbax HILIC Plus, 100 × 2.1 mm, 3.5 µm; Mobile phase: Gradient; (A) 10 mM ammonium formate, (B) 10 mM ammonium formate in 90% ACN; Flow rate: 0.2 mL/min | 100 µL urine; Centrifugation and off-line SPE with Sep-Park $C_{18}$; Elution directly into the autosampler vials | LOQ: ≤ 50 [b] | [58] |

**Table 5.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| Twenty-seven doping agents from the WADA list | *HILIC-ESI-HRMS (Q Exactive)* Stationary phase: Nucleodur HILIC, 100 × 2 mm, 1.8 µm with a Nucleodur HILIC trapping column, 20 × 2 mm, 3 µm; Mobile phase: Gradient; (A) Water, (B) ACN, (C) 0.15% glacial AA in water with 200 mM ammonium acetate; Flow rate: 0.25 mL/min | 270 µL urine; Dilution with ACN; Injection into the system | <$5\times10^3$-$10^5$ [b] | [59] |
| *Group 1:* 7-AC, 7-AF, 7-AN, AP, BZE, COD, C6G, EP, KET, MTD, MP, MDMA, MOR, M3G, M6G, O-DM-tramadol, oxycodone, pethidine, pregabalin, ritalinic acid, tramadol, zolpidem, zopiclone | *UPLC-ESI-MS/MS (QqQ)* Stationary phase: Acquity HSS T3, 100 × 2.1 mm, 1.8 µm at 50ºC; Mobile phase: Gradient; (A) 0.1% FA in water, (B) MeOH; Flow rate: 0.2 mL/min | 60 µL urine; Dilution with MeOH/water (10:90, v/v); Injection into the system | 1-60 [a] | [60] |
| *Group 2:* DMD, buprenorphine, ethylmorphine, fentanyl, MDA, 6-AM, OH-alprazolam, oxazepam, PMA, PMMA, THCA | Stationary phase: Acquity HSS T3, 100 × 2.1 mm, 1.8 µm at 50ºC; Mobile phase: Gradient; (A) 0.1% FA in water, (B) ACN; Flow rate: 0.2 mL/min | 100 µL urine; Enzymatic hydrolysis with β-glucuronidase, dilution with MeOH/water (60:40, v/v) and centrifugation | 0.2-15.0 [a] | |

**Table 5.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| BZDs, metabolites and analogues | *RRLC-ESI-MS/MS (QqQ)* Stationary phase: Hypersil GOLD perfluorophenyl, 100 × 2.1 mm, 1.9 μm with a Zorbax SB-$C_8$ pre-column, 30 × 2.1 mm, 3.5 μm; Mobile phase: Gradient; (A) 0.1% FA in water with 1 mM ammonium formate, (B) 0.1% FA in ACN with 1 mM ammonium formate; Flow rate: 0.3 mL/min | 100 μL urine; Dilution with water; Injection into the system | 0.01-0.50 [b] | [61] |
| Forty-seven conventional DOAs and forty-six NPS analytes | *UHPLC-ESI-MS/MS (QqQ)* *For positive ionization:* Stationary phase: Eclipse Plus $C_8$, 100 × 3 mm, 1.8 μm; Mobile phase: Gradient; (A) 0.1% FA in water with 5 mM ammonium formate, (B) MeOH; Flow rate: 0.3 mL/min *For negative ionization:* Stationary phase: Extend $C_{18}$, 50 × 2.1 mm, 1.8 μm; Mobile phase: Gradient; (A) 0.025% ammonium hydroxide, (B) MeOH; Flow rate: 0.3 mL/min | 1,000 μL urine; Enzymatic hydrolysis with β-glucuronidase and off-line SPE with Oasis MCX; Evaporation/reconstitution | 1-250 [b] | [62] |
| AP, MP, MDA, MDMA, MBDB, MDEA, KET, PMMA, EP, MEPH, cathinone, MCAT | *UPLC-ESI-MS/MS (QqQ)* Stationary phase: ACQUITY UPLC BEH Phenyl, 100 × 2.1 mm, 1.7 μm at 35ºC; Mobile phase: Gradient; (A) ACN, (B) 0.3% FA in water; Flow rate: 0.4 mL/min | 4,000 μL urine; Centrifugation and off-line SPE with Oasis MCX | 0.005-0.025 [b] | [63] |
| Zolpidem, zopiclone and their main urinary metabolites | *For identification:* *UHPLC-ESI-MS/MS (QqQ)* Stationary phase: Kinetex $C_{18}$, 100 × 2 mm, 2.6 μm at 40ºC; Mobile phase: Gradient; (A) water, (B) 0.1% FA in MeOH; Flow rate: 0.3 mL/min | 100 μL urine; Enzymatic hydrolysis with β-glucuronidase and centrifugation; Injection into the system | 0.5 [b] | [64] |

**Table 5.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| | *For confirmation:* *UHPLC-ESI-HRMS (Exactive)* Stationary phase: Hypersil GOLD perfluorophenyl, 100 × 2.1 mm, 1.9 μm at 40ºC; Mobile phase: Gradient; (A) 0.05% FA in water with 5 mM ammonium formate, (B) 0.05% FA in MeOH; Flow rate: 0.4 mL/min | | | |
| Synthetic cannabinoids, synthetic cathinones and conventional DOAs, (MOR, 6-AM, norbuprenorphine, buprenorphine, MDMA, THC-COOH) | *UHPLC-ESI-HRMS (TOF)* Stationary phase: Waters HSS T3, 150 × 2.1 mm, 1.8 μm with an equivalent guard column, 5 × 2.1 mm at 60ºC; Mobile phase: Gradient; (A) 0.1% FA in water with 2 mM ammonium acetate, (B) MeOH; Flow rate: 0.3 mL/min | 1,000 μL urine; Enzymatic hydrolysis with β-glucuronidase and off-line mixed-mode SPE; Evaporation/reconstitution | 0.2-60.0 [b] | [65] |
| Sixty-two common DOAs and their metabolites | *UHPLC-ESI-HRMS (QTOF)* Stationary phase: Poroshell EC-$C_{18}$, 100 × 2.1 mm, 2.7 μm; Mobile phase: Gradient; (A) 0.1% AA in water, (B) MeOH; Flow rate: 0.4 mL/min | 100 μL urine; Dilution in water and centrifugation; Injection into the system | 2.8-187.5 [b] | [66] |
| Nineteen BZDs, twelve opiates, COC and three metabolites, and three Z-drug hypnotic sedatives | *UPLC-ESI-HRMS (Q Exactive)* Stationary phase: Cortecs™ UPLC® $C_{18}$, 50 × 2.1 mm, 1.6 μm with a matching guard column, 5 × 2.1 mm; Mobile phase: Gradient; (A) 0.1% FA in ACN, (B) 0.1% FA in water with 5 mM ammonium formate: Flow rate: 0.5 mL/min | 300 μL urine; Enzymatic hydrolysis with β-glucuronidase and SLE, DCM/ isopropanol (95:5, v/v) as the solvent; Evaporation/reconstitution | 1-3 [b] | [67] |

**Table 5.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| GHB-Gluc, GHB-Sulf | *LC-ESI-HRMS (QTOF)* Stationary phase: Hypercarb, 50 × 1 mm, 3 µm; Mobile phase: Gradient; (A) 0.1% FA in water, (B) 0.1% FA in ACN: Flow rate: 0.15 mL/min | 100 µL urine; Direct injection into the system | 2 [b] (GHB-Sulf); 3 [b] (GHB-Gluc) | [68] |
| Thirty-nine new designer drugs | *LC-ESI-HRMS (QTOF)* Stationary phase: Zorbax Eclipse Plus $C_{18}$, 100 × 2.1 mm, 1.8 µm with a pre-column SecurityGuard Ultra $C_{18}$, 10 × 2.1 mm, 2 µm; Mobile phase: Gradient; (A) 5 mM ammonium formate at pH 4; (B) 0.1% FA in ACN: Flow rate: 0.4 mL/min | 200 µL urine; SALLE; Injection into the system | 1-9 [a] | [69] |
| Two hundred doping agents from the WADA list | *LC-ESI-HRMS (Q Exactive)* Stationary phase: Nucleoshell RP18, 50 × 2 mm, 2.7 µm with a Accuore Phenil/Hexyl trapping column, 10 × 3 mm, 2.7 µm; Mobile phase: Gradient; (A) 5 mM ammonium acetate with 0.1% AA, (B) ACN; Flow rate: 0.4 mL/min | 90 µL urine; Direct injection into the system | <0.05-$10^4$ [b] | [70] |
| Different drugs, including MOR, C6G, COD, AP, diazepam | *LC-ESI-HRMS (Q Exactive)* Stationary phase: TF Accucore PhenylHexyl, 100 × 2.1 mm, 2.6 µm; Mobile phase: Gradient; (A) 2 mM ammonium formate with 0.1% FA, (B) 2 mM ammonium formate in ACN:MeOH (50:50, v/v; 1% water) with 0.1% FA, (C) propanol:ACN (50:50, v/v); Flow rate: 0.05-0.5 mL/min | 200 µL urine; On-line extraction by turbulent flow chromatography<br><br>100 µL urine; Dilute-and-shoot | 0.1-100.0 [b]<br><br>1-100 [b] | [71] |

**Table 5.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| 6-AM, AP, MP, MDA, MDMA, MDEA, PCP, MOR, BZE, COD and fifteen synthetic cathinones | *Mixed-mode UHPLC-ESI-HRMS (TOF)*<br>Stationary phase: Perkin-Elmer PFP, 150 × 2.1, 2.7 μm; Flow rate: 0.3 mL/min<br>• *RPLC conditions:*<br>Mobile phase: Gradient; (A) 10 mM ammonium formate in ACN:water (1:9, v/v), (B) 10 mM ammonium formate in ACN:water (9.5:0.5, v/v)<br>• *HILIC conditions:*<br>Mobile phase: Isocratic, 5% A and 95% B; (A) 10 mM ammonium formate in ACN:water (1:9, v/v), (B) 10 mM ammonium formate in ACN:water (9.5:0.5, v/v) | 500 μL urine;<br>Dilution with 200 mM ammonium formate or ACN and off-line SPE with a silica, reversed-phase/strong cation exchange cartridge | 1-67 [b] | [72] |
| Meperidine, *cis*-tramadol, MOR, COD, MTD, hydrocodone, oxymorphone, oxycodone, naloxone, fentanyl, naltrexone, buprenorphine | *Mixed-mode UHPLC-ESI-HRMS (TOF)*<br>Stationary phase: Perkin-Elmer PFP, 150 × 2.1, 2.7 μm; Flow rate: 0.3 mL/min<br>• *RPLC conditions:*<br>Mobile phase: Gradient; (A) 5 mM ammonium formate in ACN:water (1:9 v/v), (B) 5 mM ammonium formate in ACN:water (9.5:0.5, v/v)<br>• *HILIC conditions:*<br>Mobile phase: Isocratic, 10% A and 90% B; (A) 5 mM ammonium formate in ACN:water (1:9 v/v), (B) 5 mM ammonium formate in ACN:water (9.5:0.5, v/v) | Off-line SPE with a silica, reversed-phase/strong cation exchange cartridge | 3-104 [b] | [73] |

**Table 5.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| Clonazolam, meclonazepam, nifoxipam, and metabolites | *Nano-LC-nanoESI-HRMS (targeted MS²)* Stationary phase: PePMap RSLC Acclaim $C_{18}$, 150 mm × 50 μm, 2 μm at 40ºC; Mobile phase: Gradient; (A) 0.1% FA in water, (B) 0.1% FA in ACN; Flow rate: 0.0003 mL/min<br><br>*UPLC-ESI-HRMS (Q Exactive)* Stationary phase: YMC UltraHT Hydrosphere C18, 100 × 2 mm, 2 μm with a guard column YMC Hydrosphere C18 at 60ºC; Mobile phase: Gradient; (A) 0.005% FA in water with 10 mM ammonium formate, (B) 0.005% FA in MeOH (10:90 v/v) with 10 mM ammonium formate; Flow rate: 0.5 mL/min | 50 μL urine; Dilution with internal standard solution and centrifugation; Injection into the system | – | [74] |

See Appendix I for the key to abbreviations.
[a] Standard samples.
[b] Urine samples.

Most of the published methods for the determination of DOAs by LC-MS, collected in the review papers cited above and Table 5 [54-74], use silica-based materials bonded with $C_{18}$ alkyl chains as the stationary phase and gradients of acetonitrile or methanol in aqueous formic acid or acetic acid solutions as the mobile phase, even though polar analytes elute very early and are more prone to interference from matrix components. In most cases, a hydrolysis step is carried out using β-glucuronidase before urine sample preparation to cleave the glucuronide bindings. The glucuronide conjugates formed are highly polar molecules and cannot be easily retained in reversed-phase LC (RPLC), thus hindering the quantitative ability of the analytical method. However, enzymatic hydrolysis has its own associated challenges. The enzyme must be removed from the specimen prior to analysis to halt its reaction with the target compounds. In addition, an increase in the column backpressure can occur due to incomplete removal of the enzyme prior to injecting the sample onto the column [75].

Hydrophilic interaction LC (HILIC) represents an alternative approach to the widely employed RPLC, offering several advantages for the analysis of polar analytes, including DOAs in urine {Table 5 [57-59]}. These advantages include the ability to provide an orthogonal separation mechanism which can resolve analytes not separated under RPLC conditions, as well as providing additional identification. Within this context, both parent drugs and glucuronide metabolites, particularly different opioid compounds and their glucuronide conjugates, have been successfully determined together without performing any enzymatic hydrolysis [57, 58]. Using HILIC can also mitigate the need for an evaporation and reconstitution step during the SPE procedure, considerably reducing the sample preparation time [58]. Görgens *et al.* [59] established a fast analysis method based on the direct injection of diluted urine to quantify twenty-seven doping agents from the World Anti-Doping Agency (WADA) list, including stimulants and narcotics, using a zwitterionic HILIC stationary phase. Moreover, as the HILIC mobile phase has a low aqueous composition, the MS response is often improved, thus achieving lower LODs. However, despite these versatile benefits, HILIC columns require more equilibration than RPLC columns. At least three blank urine samples have to be injected to precondition the HILIC column before the first run of the sequence.

To further improve the resolving power and/or throughput in RPLC, ultra-high performance LC (UHPLC), also named ultra-performance LC (UPLC) or rapid resolution LC (RRLC), which employs columns packed with sub-2 μm particles under very high-pressure conditions, has become a powerful separation tool for DOAs analysis in urine. As can be seen in Table 5, the $C_{18}$ columns are the most widely used in UHPLC [60, 62, 64-67], but some other stationary phases, such as perfluorophenyl [61, 64], $C_8$ [62] and phenyl [63], have also been successfully used for the analysis of DOAs in urine.

Mixed-mode chromatography, in which a stationary phase exhibits both RPLC and HILIC properties, has recently been reported for the determination of the basic compounds in the SAMHSA-5 panel and synthetic cathinones [72] and several opioid analytes [73] in urine samples. The stationary phase used in these research works was pentafluorophenyl (PFP). Unlike RPLC and HILIC, mixed-mode chromatography uses a single column and the same mobile phase solvents in both RPCL and HILIC modes. This is possible as, at low concentrations of organic solvent, the RPLC mechanism predominates, while, at high concentrations of organic solvent, the retention is mainly controlled by the HILIC mechanism. For example, Figure 9

illustrates the extracted ion chromatograms of an SPE extract of a spiked urine blank sample using a PFP column in both the RPLC and HILIC [72]. As can be observed in the figure, complementary separations were obtained as shown by the significantly different retention order for the analytes of interest. All the compounds were fully resolved by a combination of both techniques.

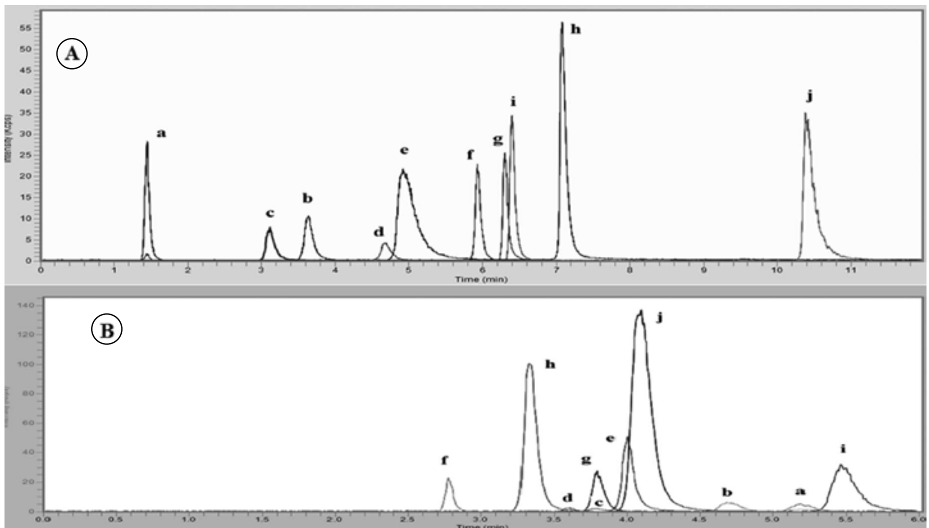


**Figure 9.** Extracted ion chromatograms of a direct SPE extract of synthetic urine containing 2,000 ng/mL each of a) morphine, (b) codeine, (c) amphetamine, (d) 3,4-methylenedioxyamphetamine, (e) methamphetamine, (f) 6-acetylmorphine, (g) 3,4-methylenedioxymethamphetamine, (h) 3,4-methylene-dioxyethylamphetamine, (i) benzoylecgonine and (j) phencyclidine. (A) RPLC separation and (B) HILIC separation on an SPP PFP column. Reproduced from [72] with the permission of The Royal Society of Chemistry, copyright 2015.

With respect to sample preparation, as reflected in the review papers considered here [42-53] and Table 5 [54-74] included in this section, LLE and SPE procedures are most commonly used for the isolation of DOAs from the urine matrix. The use of these extraction approaches usually requires a pretreatment step, such as the adjustment of the sample pH, protein precipitation or centrifugation. DOAs such as opiates/opioids and benzodiazepines are excreted in urine as conjugated metabolites, mainly as the glucuronide. In this respect, cleavage of conjugates is often performed by acidic or enzymatic hydrolysis [60, 62, 64, 65, 67].

Recently, magnetic SPE, a novel mode of SPE based on the use of magnetic adsorbents, has been combined with LC for the extraction and determination of

certain DOAs in urine samples [54, 55], providing satisfactory results. For example, Taghvimi and Hamishehkar [55] used laboratory-made carbon-coated magnetite ($Fe_3O_4$) particles to extract ephedrine and methamphetamine from urine prior to analysis by LC, achieving recoveries above 97%. The proposed methodology proved to be simple and fast since it consisted of the following steps: adding the particles to the sample, allowing extraction to take place (2 minutes), collecting the particles using an external magnet, placing the particles in a proper solvent and, finally, allowing desorption to take place (5 minutes). Nevertheless, the fact that these particles are not commercially available and must be synthesized by the users might have an influence on the reproducibility of the method, which may be considered as a drawback of this approach.

The use of ionic liquids has also attracted great interest due to their physicochemical properties and also because they are green solvents, therefore, being ideal for replacing conventional organic solvents. Ionic liquids have been successfully used in dispersive liquid-liquid microextraction coupled with LC for the methamphetamine analysis in urine [56]. Using 1-octyl-3-methylimidazolium hexafluorophosphate, enrichment factors up to 220-fold and acceptable recoveries (>80%) were obtained, resulting in an LOD of 1.7 ng/mL. These results clearly open opportunities for ionic-liquid-based procedures to eventually be used in laboratories testing for DOAs.

Another interesting approach for sample preparation is the usage of a trapping column before LC separation, which enables the direct injection of native urine specimens, while providing an on-line clean-up and preconcentration of the sample, and preventing the analytical system from interfering matrix contaminants. Such a strategy was proposed for screening more than 200 analytes of various classes of doping agents included on the 2016 WADA list in urine, including narcotics and stimulants [70]. The orthogonality between a phenyl-hexyl trap column and $C_{18}$ separation column enabled urine analysis without any time-consuming hydrolysis or further purification steps, reaching LODs in the range of 0.05-25.00 ng/mL, except for one compound.

Helfer *et al.* [71] developed and compared the analytical performance of on-line extraction by turbulent flow chromatography (TurboFlow) and a dilute-and-shoot approach for the extraction from urine of different analytes, including DOAs such as morphine, codeine-6-glucuronide, amphetamine, codeine and diazepam, prior to determination by LC. The TurboFlow approach, as it is known, consists of using flow rates above 1.2 mL/min using 0.5 mm internal diameter (i.d.) columns packed with

large particles of 30-50 μm. In such a way, larger molecules present in the sample diffuse more slowly into the pores of the particles than small molecules and are therefore less retained. Dilute-and-shoot, on the other hand, consists of diluting the sample with an appropriate solvent and then injecting it into the instrument without any further sample treatment, thereby considerably reducing the overall analysis time. Under the optimal conditions, TurboFlow showed low recoveries for hydrophilic compounds, such as morphine (39%) and amphetamine (44%), high signal suppression and LOD values in the range 0.1-100.0 ng/mL. In contrast, dilution provided high recoveries (around 100% for almost all the compounds), low signal suppression, but with higher LODs (1-100 ng/mL). Both procedures proved to be fast and suitable for the intended purpose (less than 7 minutes), although TurboFlow requires additional instrumentation, a fact that may limit its widespread application.

As regards the identification and/or quantification of DOAs in urine, the vast majority of the published LC-based methods use MS/MS [50-52, 57, 60-64], although other detectors like UV detection are still used for screening and quantification [54-56]. LC-MS/MS assays are generally divided in two groups: targeted methods for the detection of a limited number of compounds (e.g., for confirmation of a positive immunoassay result) and non-targeted methods intended for a general unknown screening aiming to identify the possible substances present in a donor's sample. In addition, targeted methods can give quantitative information [50-52].

For targeted MS/MS methods, triple quadrupole (QqQ) and quadrupole ion trap (QTrap), both operating in multiple reaction monitoring (MRM) mode, are the most widely used low-resolution MS analysers [50-52, 57, 60-64]. Due to the very high sensitivity and specificity afforded by these instruments, different classes of DOAs (e.g., stimulants, narcotics, depressants) have been analysed after a simple dilute-and-shoot procedure [50-52, 60, 61]. However, under these conditions, the ionization source is more vulnerable to matrix effects caused by co-eluting interfering components. To avoid false positives, most of the published methods monitored at least two MRM transitions per analyte.

Despite the considerable success of the MRM mode, due to the scanning speed and dwell time restrictions, only a limited number of targets can simultaneously be analysed, while other non-targeted analytes that might also be present in the sample would not be detected. To overcome this issue, non-targeted LC-MS/MS techniques with high-resolution MS (HRMS), using either time-of-flight (TOF) or Orbitrap

technology [50, 51, 53, 59, 65-67, 69, 70, 74], have been proposed for the broad-spectrum screening of DOAs in urine. The LC-HRMS approaches proposed enable screening for hundreds and even thousands of compounds in urine, including new designer drugs (e.g., phenethylamines, amphetamines, cathinones, piperazines) and common DOAs (e.g., opiates, hallucinogens, stimulants, sedative-hypnotics), with LODs that are generally in line with the cut-off levels in urine (listed in Table 4) within one chromatographic run. In addition, HRMS instruments have also been successfully used in recent years for target analysis (quantitative analysis) [50, 51, 53, 58, 64, 68, 69, 72, 73].

Within this context, HRMS devices seem to be suitable for both qualitative and quantitative analysis of DOAs in urine and will increasingly replace immunoassays in qualitative analysis and QqQ devices in quantification, offering an all-in-one solution [76, 77]. Currently, the rather high cost of these instruments is suppressing interest in them, but they will gain greater acceptance when low-budget solutions become readily available.

Ionization of DOAs and their metabolites has mainly been performed using electrospray ionization (ESI) in both targeted and non-targeted approaches [50-53, 57-74]. Because most DOAs have basic chemical properties, positive ionization mode has generally been applied [50, 51, 57, 58, 61, 63-65, 67, 69, 72-74]. The negative mode has also been used in combination with the positive mode in an attempt to widen the spectrum of the analytes covered by the respective methods [50, 51, 53, 59, 60, 68]. In a few occasions, when QqQ, QTrap and TOF were used as the mass analysers [50, 51, 60, 62], the positive and negative ionization was performed in two separate injections, thereby increasing costs and overall analysis time. In contrast, when Orbitrap technology was used (including Exactive and Q Exactive) [51, 53, 59, 70, 71], the fast polarity switching afforded by these instruments enabled both positive and negative scans within one analytical run. These results show that Orbitrap-based approaches may be useful for drug analysis in the case of the workplace, doping controls or reissuing driving licenses, in which a general profile of drug use is required or in cases in which drug use is suspected but unproven.

Moreover, miniaturization is one of the emerging trends in modern analytical chemistry. Within this context, nano-LC has successfully been applied for testing DOAs in various biological matrices [78]. In a recent paper, nano-LC-HRMS was used to identify designer benzodiazepines and metabolites in urine samples collected

from drug abusers [74]. The authors also analysed the target compounds in urine by UHPLC-HRMS and the results showed increased MS signal intensities using nano-LC compared to UHPLC, as a consequence of higher sensitivity. In fact, the literature reports that reducing the LC flow rate results in increased sensitivity due to better desolvation of the analytes [76]. However, in spite of this great benefit, nanoflow methods generally do not offer high throughput and, nowadays, this is a limitation for their widespread use.

*GAS CHROMATOGRAPHY*

GC is generally coupled to MS through electron impact (EI) ionization, and this approach has been particularly useful in toxicological laboratories for several decades due to the relatively low-cost instrumentation compared to LC and also the commercial availability of several DOAs identification libraries. However, as is well documented, in the analysis of DOAs by GC, derivatization of the analytes is necessary prior to instrumental analysis and this additional step can be considered as a drawback, since it clearly lengthens the analysis time and may complicate the sample pretreatment. As a consequence, LC has become increasingly used in DOAs testing laboratories, although interesting approaches using GC-MS also can be found in the literature. As in the case of LC, the applicability of GC in urine drug testing has been discussed in several recent review articles, some of them are listed here [42-48, 79], including the sample preparation, derivatization reagents, GC column and detection details. These publications cover papers published up to 2015. With this in mind, a number of the most relevant research works reported during and since this year will be highlighted here {Table 6 [80-89]}.

As is well known, derivatization is one the most important steps during sample preparations in GC analysis. Further details on derivatization for the analysis of DOAs in biological matrices, including urine, by GC-MS have been extensively discussed in various recent documents [90-92]. In short, the procedures collected in these review papers show that silylation and acylation are commonly applied chemical derivatization reactions in urine drug testing.

Most of the derivatization processes proposed in the literature typically require reaction times ranging from 30 minutes up to several hours at elevated temperatures (generally around 60ºC). Therefore, derivatization clearly represents the rate-limiting

**Table 6.** GC-based methods for the determination of DOAs in urine.

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| α-PVP and its metabolites | *GC-EI-MS* Column: Macherey-Nagel Optima 5MS Accent, 30 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 5,000 μL urine; Hydrolysis with concentrated HCl (30 min, 100ºC); LLE with EtAc/DCM/isopropanol (3:1:1, v/v) and MAD with AA and Pyr; Evaporation/reconstitution | – | [80] |
| Synthetic cannabinoids and metabolites | *GC-EI-MS* Column: 5% phenyl methyl siloxane, 15 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 5,000 μL urine; Enzymatic hydrolysis with β-glucuronidase, LLE with 1-chlorobutane and derivatization with TFA and HFIPOH (15 min, 70ºC); Evaporation/reconstitution | 0.1 [a] | [81] |
| Twenty-two amphetamine-type synthetic drugs | *GC-EI-MS* Column: TG-5MS, 15 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 2,000 μL urine; LLE with cyclohexane and derivatization with PFBCI; Evaporation/reconstitution | 0.06-2.50 [b] | [82] |
| Six synthetic cathinones | *GC-EI-MS* Column: HP-5MS, 15 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 2,000 μL urine; Off-line SPE with SPEC® DAU cartridge and derivatization with HFBA and EtAc; Evaporation/ reconstitution | 5-20 [b] | [83] |
| MP, KET | *GC-EI-MS* Column: DB-5MS, 30 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 4,000 μL urine; Magnetic SPE with o-MWNTS; Injection into the system | 0.044 [a] (MP); 0.024 [a] (KET) | [84] |
| Three ATS | *GC-EI-MS* Column: HP-5MS, 30 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 5,000 μL urine; DLLME; Injection into the system | 0.05-0.10 [a] | [85] |
| MP, AP, MTD, MDMA, MDA, MCAT, KET, meperidine | *GC-EI-MS* Column: HP-5MS, 30 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 1,000 μL urine; HF-LPME or UA-LDS-DLLME; Injection into the instrument | 0.5-5.0 [b] | [86] |

**Table 6.** (Continued).

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| Six cathinone-type synthetic drugs | *GC-EI-MS/MS (IT)* Column: BPX5, 30 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 10-40 μL urine; Oximation with hydroxylamine-hydrochloride and Pyr (60 min, 100°C) and silylation with MSTFA (30 min, 70°C); Dilution and injection into the system | LOQ: $15\times10^{3}$-$24\times10^{3}$ [b] | [87] |
| KET, NKET | *GC-EI-MS/MS (QqQ)* Column: HP-5MS, 30 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 100 μL urine; MEPS; Evaporation/reconstitution | 5 [b] | [88] |
| Four designer BZDs | *GC-EI-MS/MS (QqQ)* Column: DB-5MS, 30 m × 0.25 mm i.d., 0.25 μm f.t.; Carrier gas: helium | 1,000 μL urine; UA-LDS-DLLME; Injection into the system | 1-3 [b] | [89] |

See Appendix I for the key to abbreviations.
[a] Standard samples.
[b] Urine samples.

step of sample analysis. Within this context, microwave-assisted derivatization protocols have been developed and demonstrated to enable a reduction in the time required for derivatization down to a few minutes [91]. For example, Grapp *et al.* [80] carried out the acylation of α-pyrrolidinovalerophenone and its metabolites with acetic anhydride and pyridine in a microwave oven at 450 W for 5 minutes. The GC-EI-MS analysis in urine collected from a real case revealed the presence of all the compounds investigated, proving that derivatization had successfully occurred under the conditions selected. However, despite the great benefits, microwave-assisted derivatization procedures need specific instrumentation, which, at the present, limits their widespread use in routine analysis.

As mentioned earlier, extraction of DOAs from urine samples is usually achieved by LLE or SPE, despite the fact that these procedures require relatively large volumes of solvents [80-83]. Furthermore, great attention has been paid in recent years to modern microextraction techniques, since they involve the reduction of organic solvent volumes with the subsequent production of less toxic residues. In this respect, a magnetic SPE procedure combined with GC-EI-MS for the determination of two

DOAs, methamphetamine and ketamine, in urine was presented by Zhang *et al.* [84]. Using oxide multi-walled carbon-nanotubes as the adsorbent, the extraction was completed within 10 minutes using 100 μL of organic solvent with satisfactory recoveries (76.3% and 86.3% for methamphetamine and ketamine, respectively) and good values of relative standard deviations (RSDs, <10.0% in both intra-day and day-to-day experiments).

Moreno *et al.* [88], meanwhile, reported an analytical procedure based on microextraction on packed sorbent followed by GC-EI-MS/MS analysis for the determination of ketamine and its major metabolite, norketamine, in urine. This microextraction technique has proven to be suitable for urine handling, enabling the use of small sample volumes (250 μL), which can be critical when specimen availability is limited, as well as reduced solvent volumes (100 μL). Furthermore, no derivatization step was needed to accomplish the analysis, making this strategy less laborious and time-consuming.

Dispersive liquid-liquid microextraction (DLLME) is another miniaturized technique that has gained considerable interest in the analysis of DOAs. A method based on this approach and GC-EI-MS was developed for the determination of three amphetamine-type stimulants in urine [85]. With high recoveries (82.1-104.2%) and good values of RSDs (<9.0% in both intra-day and day-to-day experiments), the method has proven to be a highly feasible option for forensic analysis. However, in spite of the good analytical results achieved, the authors employed larger volumes of organic solvent compared with the methodologies described above [84, 88] (1,000 μL of methanol as disperser solvent plus 300 μL of dichloromethane as extractor solvent), which can be considered as a drawback of this strategy, since the use of eco-friendly techniques is the current trend nowadays.

Other microextraction techniques based on HF-LPME and ultrasound-assisted low-density solvent dispersive liquid-liquid microextraction (UA-LDS-DLLME) have been used in conjunction with GC separation for urine drug testing [86]. According to the authors, both approaches proved to be very simple, rapid and environmentally friendly techniques (in HF-LPME, 10 μL of toluene was used and, in UA-LDS-DLLME, 100 μL). For the analysis of spiked urine samples, recoveries of 79.3-98.6% with RSDs of 1.8-4.2% were obtained when using HF-LPME, and recoveries of 80.6-103.4% with RSDs of 2.4-4.8% were obtained when using UA-LDS-DLLME. These data reveal that there was no significant difference between the analytical

performances of both methods. However, as can be seen in Figure 10, a cleaner chromatogram was obtained after HF-LPME extraction in comparison to the one obtained for UA-LDS-DLLME, which indicates that HF-LPME has a better sample clean-up effect.

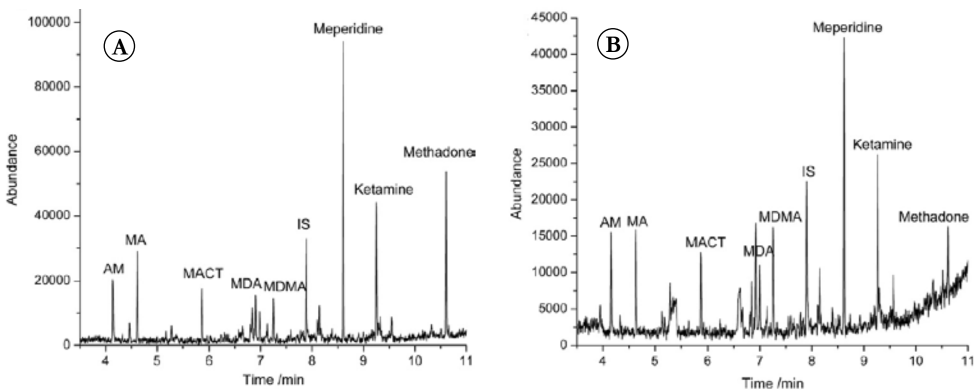


**Figure 10.** Full scan chromatograms obtained with spiked urine samples under the optimized conditions of the proposed methods based on (A) HF-LPME and (B) UA-LDS-DLLME. Analytes concentration spiked, 500 ng/mL; internal standard: 2-MeOPP, 15,000 ng/mL. Reproduced from [86] with the permission of Elsevier B.V., copyright 2015.

With respect to the detection system, single quadrupole MS operated in the full scan mode and in the single ion monitoring mode for identification and quantification, respectively, has been the most widely employed in the analysis of DOAs in urine samples by GC [80-82, 84-86]. LODs in the range of 0.024-5.000 ng/mL were achieved, which are far below the cut-off levels in urine (listed in Table 4).

MS/MS has also been reported in combination with GC for the same purpose. The use of MS/MS detection offers additional specificity with secondary fragmentations, and thus may circumvent co-elution problems. For example, Molnár *et al.* [87] developed an analytical method based on GC-EI-ion trap MS/MS operated in the MRM mode for the quantification of several synthetic cathinones in urine samples without preliminary extraction of the drugs. The urine samples were subjected to centrifugation, evaporation to dryness and two-step derivatization, before then being directly injected into the system for analysis. The LOQs were 15,000-24,000 ng/mL. This method proved to be time-, work-, cost- and solvent-effective, although the sensitivity achieved is more suitable when high concentrations are expected to be found, e.g., in overdose cases, reasonable suspicions in the workplace, therapeutic

drug monitoring or drug rehabilitation programs. In another study, a GC-EI-(QqQ)MS/MS method operated in the MRM mode to determine four designer benzodiazepines in urine was established [89]. The urine samples were subjected to a simple and fast procedure based on UA-LDS-DLLME. Figure 11 shows the high selectivity and sensitivity of this methodology for a spiked urine sample and an individual's urine sample after DLLME, achieving LOQs in the range of 3.5-10.0 ng/mL.

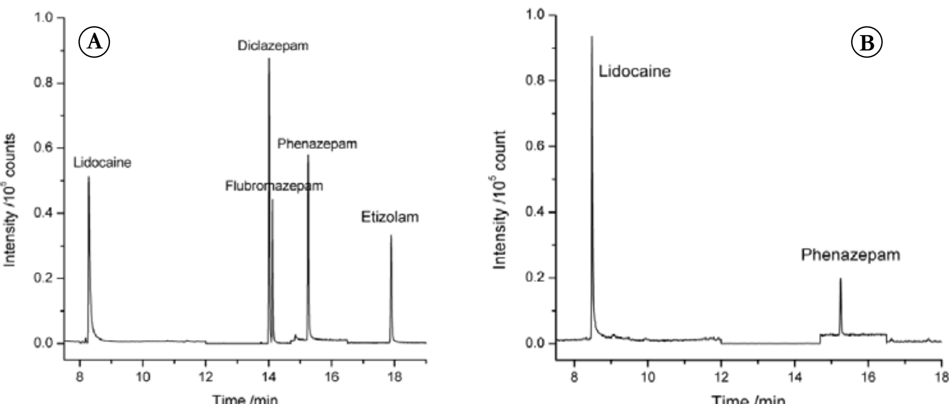


**Figure 11.** (A) MRM chromatogram obtained with spiked urine samples by DLLME under the optimized conditions. Concentration of each drugs: diclazepam, 150 ng/mL; others, 250 ng/mL; internal standard: lidocaine, 5,000 mg/mL. (B) MRM chromatogram of a suspect's urine sample. Reproduced from [89] with the permission of Elsevier B.V., copyright 2017.

*SUPERCRITICAL FLUID CHROMATOGRAPHY*

In addition to LC and GC, supercritical fluid chromatography (SFC) has also been investigated very recently, albeit not widely, for the analysis of some DOAs in urine {Table 7 [93-95]}.

This technique has made a remarkable comeback among the analytical chemistry community due to improvements in instrumentation (better performance, reliability and robustness) and column technology, allowing the use of columns packed with fully porous sub-2 μm particles, as well as columns packed with superficially porous sub-3 μm particles (fused-core technology) [96-99]. Thus, the performance of such instruments rivals conventional UHPLC.

**Table 7.** SFC-MS methods for the determination of DOAs in urine.

| *Substance* | *Separation and detection* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| Cannabinoids and metabolites | *SFC-UV* Stationary phase: Zorbax Rx-SIL, 150 × 4.6 mm, 5 µm at 40ºC; Mobile phase: Isocratic; $CO_2$: ACN (93:7, v/v); Flow rate: 2.5 mL/min | 1,000 µL urine; LLE with ACN and centrifugation; Evaporation/ reconstitution | 150-520 [b] | [93] |
| One hundred and ten doping agents from the WADA list | *UHPSFC-ESI-MS/MS (QqQ)* Stationary phase: Acquity UPC² BEH, 100 × 3 mm, 1.7 µm at 40ºC; Mobile phase: Gradient; (A) $CO_2$, (B) MeOH with 10 mM ammonium formate and 2% water; Flow rate: 1.5 mL/min | 100 µL urine; Dilution with water:ACN (25:75, v/v); Injection into the system | 0.002-15.000 [a] | [94] |
| | *UHPLC-ESI-MS/MS (QqQ)* Stationary phase: Acquity BEH $C_{18}$, 50 × 2.1 mm, 1.7 µm at 40ºC; Mobile phase: Gradient; (A) ACN, (B) 0.1% FA in water with 2% ACN; Flow rate: 0.5 mL/min | | 0.003-1.500 [a] | |
| Synthetic cannabinoids and metabolites | *UHPSFC-ESI-MS/MS (QqQ)* Stationary phase: Acquity UPC² BEH, 100 × 3 mm, 1.7 µm at 40ºC; Mobile phase: Gradient; (A) $CO_2$, (B) MeOH with 0.3% ammonia; Flow rate: 2 mL/min | 450 µL urine; Enzymatic hydrolysis with β-glucuronidase and centrifugation; Evaporation/reconstitution | LOQ: 0.04-0.40 [b] | [95] |
| | *UHPLC-ESI-MS/MS (QqQ)* Stationary phase: Acquity UPLC® BEH $C_{18}$ at 60ºC, 50 × 2.1 mm, 1.7 µm; Mobile phase: Gradient; (A) 5 mM ammonium formate (pH 10.2), (B) MeOH; Flow rate: 0.4 mL/min | | | |

See Appendix I for the key to abbreviations.

[a] Standard samples.

[b] Urine samples.

In the aforementioned context, methods involving UHPLC-(QqQ)MS/MS and UHPSFC-(QqQ)MS/MS have been developed for qualitative and/or quantitative analysis of several doping agents on the WADA list (including stimulants and narcotics) [94] and a selection of synthetic cannabinoids and metabolites [95] in urine. The authors of these studies reported that very diverse retentions and selectivities were obtained in UHPLC and UHPSFC. In consequence, co-elutions observed in UHPLC were resolved in UHPSFC and vice versa, demonstrating the complementarity of these two analytical strategies. The sensitivities achieved were good with both approaches, with LOD and LOQ values (listed in Table 7) below the cut-off levels in urine [39, 41].

In conclusion, SFC-MS represents a viable alternative to LC-MS for bioanalytical applications. However, the technology is not mature enough to be routinely employed in toxicological laboratories testing.

*CAPILLARY ELECTROPHORESIS*

As well as the techniques mentioned above, CE-based methods have also been applied to determine DOAs and their metabolites in urine samples [46, 78, 100]. In the present Doctoral Thesis, Section 1.3. contains an in-depth discussion regarding the state of the art and the latest trends of the CE-based strategies proposed for the analysis of DOAs in a wide variety of matrices, including urine. Therefore, the reader is referred to this section.

*NOVEL APPROACHES*

High-throughput screening is essential for clinical and forensic toxicology, crime-scene investigations, workplace drug testing and doping control. While hyphenated chromatography-MS (e.g., LC-MS, GC-MS) remains the approach of choice for such a task, ambient MS techniques {summarized in Table 8 [101-108]}, such as direct analysis in real time (DART) [101-104], surface-assisted laser desorption/ionization (SALDI) [105, 106], desorption atmospheric pressure photoionization (DAPPI) [107] and desorption electrospray ionization (DESI) [107, 108], have also been proposed for DOAs testing in urine. These offer the advantage of analysing sample in its native status with minimal or even no sample pretreatment.

**Table 8.** Ambient MS methods for the determination of DOAs in urine.

| *Substance* | *Analytical technique* | *Sample pretreatment* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|
| DMAA | *DART-HRMS (TOF)* | – | – | [101] |
| COC, MTD | *DART-MS/MS (QqQ)* | Thin-film SPME ($C_{18}$-polyacrylonitrile-coated meshes) | 0.5 [b] | [102] |
| Synthetic cathinones and metabolites | *DART-HRMS (Exactive)* | SPME | LOQ: 2 [b] | [103] |
| COC and metabolites | *DART-HRMS (TOF)* | MEPS | 4.0-23.7 [b] | [104] |
| COC, MDMA, MTD | *SALDI-HRMS (MALDI-TOF-TOF)* | 50 µL urine; Porous silicon micro-particles | 20 [a] | [105] |
| MTD | *SALDI-HRMS (MALDI-TOF-TOF)* | 200 µL urine; LLE with chloroform and porous silicon micro-particles | 19.5 [b] | [106] |
| Four BZDs and four opioids | *DAPPI-MS*<br>*DAPPI-MS/MS (IT)* | PMMA* sampling surface | $200\text{-}9\times10^{4}$ [b]<br>$75\text{-}7\times10^{4}$ [b] | [107] |
| | *DESI-MS*<br>*DESI-MS/MS (IT)* | PTFE sampling surface | $5\times10^{3}\text{-}10^{5}$ [b]<br>$600\text{-}7\times10^{4}$ [b] | |
| COC, BZE, COE, meprobamate, norfentanyl, MTD, EDDP | *For screening:* *DESI-HRMS (TOF)* *For quantification:* *DESI-MS/MS (QqQ)* | SPME ($C_{18}$) | 10-160 [b] | [108] |

See Appendix I for the key to abbreviations.
[a] Standard samples.
[b] Urine samples.

Recently, DART has been used in combination with HRMS using a TOF mass analyser for the direct analysis of urine to detect dimethylamylamine, which is a stimulant banned by the WADA, without preparation, preconcentration or processing procedures [101]. However, under these conditions, other major components in the urine sample, specifically urea and creatinine, were also identified, although the authors claimed that the matrix effects did not preclude the detection of the target compound.

As is now well known, when it comes to MS techniques, any interference with the ionization of the target compounds results in decreased intensity, so any sample preparation that selectively removes these substances has the potential to benefit the assay [109-111]. Within this context, solid-phase microextraction (SPME) has proven to be a good approach to use as pretreatment step in DART-MS analysis. Rodríguez-Lafuente *et al.* [102] used a $C_{18}$-polyacrylonitrile coated mesh immersed in urine samples to extract cocaine and methadone in a technique they called thin-film SPME, while LaPointe *et al.* [103] immersed $C_{18}$ SPME fibres in urine specimens to detect bath salts and metabolites. In both studies, once the solid phase was loaded with the analytes of interest, it was positioned directly in front of the DART-MS source for analysis. Figure 12 shows the DART-MS spectra obtained for a urine sample spiked with cocaine and methadone at a concentration of 0.5 ng/mL each, in the first case, directly analysing the specimen and, in the second, after extraction with a SPME-coated mesh approach [102]. From the figure, it can be observed that the use of the SPME together with DART-MS contributed to a significant enhancement of the signal intensities, thereby allowing the detection of concentrations down to the ng/mL range in urine (e.g., LaPointe *et al.* [103] detected a concentration of 0.2 ng/mL for 3,4-dimethylmethcathinone).

In addition to DART, SALDI-HRMS has recently been demonstrated as suitable to be used for detecting DOAs, such as methadone, cocaine and 3,4-methylenedioxymethamphetamine, in several biological matrices, including urine [105]. Although the authors claim that direct analysis was carried out without the need for extensive sample treatment, properly functionalized nanostructures substrates were prepared for the purpose of enriching the analytes of interest. Once fabricated, these microparticles, were immersed into the urine sample containing the drugs and left to perform the intended extraction. After 15 min, 2 μL of the microparticles in solution were removed using a micropipette from the urine sample and pipetted onto a standard matrix-assisted laser desorption/ionization target plate for SALDI-MS analysis. Later, a similar approach to the one previously described was successfully developed and applied for the quantitative analysis of methadone in urine collected from patients who were enrolled in an opioid replacement program [106]. This methodology may offer an alternative method, although it should be investigated further to assess other DOAs before its potential use in routine laboratories testing.

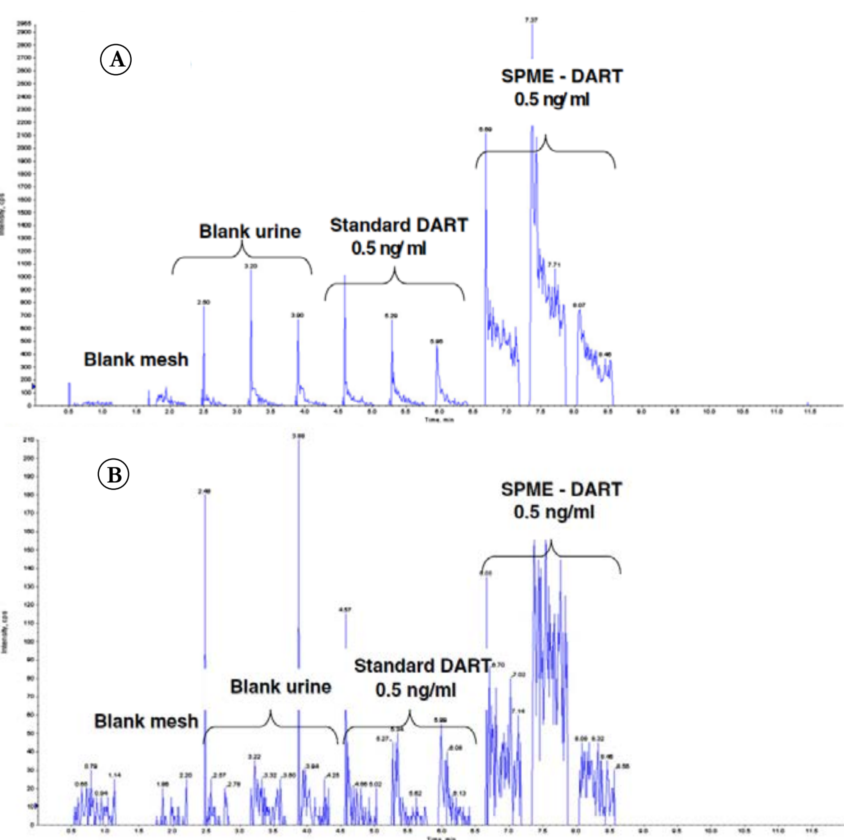


**Figure 12**. Increment of signal intensity observed when employing SPME-coated mesh extraction before DART for (A) methadone and (B) cocaine (0.5 ng/mL in urine). Blank urine is blank urine deposited in the bare mesh, standard DART 0.5 ng/mL is spiked urine deposited in the bare mesh, SPME DART-MS 0.5 ng/mL is spiked urine extracted with the proposed method, blank mesh is the bare stainless-steel mesh. Reproduced from [102] with the permission of Springer-Verlag Berlin Heidelberg, copyright 2013.

DAPPI and DESI with a single-quadrupole MS have also been studied for the direct analysis of benzodiazepines and opioids in urine samples [107]. The authors reported that DAPPI provided ten times better sensitivity and longer operation without the need to clean the mass spectrometer, indicating better matrix tolerance in the case of DAPPI compared to DESI. However, in DAPPI, the urine matrix may affect the ionization mechanism. Moreover, in the MS/MS mode, DAPPI provided better sensitivity than DESI for the investigated drugs in urine, achieving LODs in the range of 75-700 ng/mL, except for morphine (LOD of 70,000 ng/mL). The LODs achieved were in accordance with the cut-off levels in urine [39, 41], except for morphine, although the LODs for benzodiazepines and opioids from urine with LC-MS(/MS)

typically range from the low ng/mL level to the low μg/mL level, providing about one to two orders of magnitude better sensitivity than DAPPI [50, 51, 53, 57, 60, 61, 64, 65, 67].

Ambient MS methods proved to be fast direct analysis tools for DOAs testing in urine. Little or no sample preparation has been used before MS analysis, although the combination of ambient techniques with sample treatment procedures may be necessary to reduce matrix effects and improve LODs. Thus, it should always be carefully considered and studied whether the sample treatment steps can be bypassed without decreasing the performance of the method, especially in cases in which a large number of biosamples must be analysed, due to the contamination of the ion source and the mass spectrometer.

### Interpretation of results

Like any laboratory test, urine drug tests can yield false-positive or false-negative results. A false-positive result (namely, detecting a drug of abuse when the individual did not consume) is more likely to occur on a screening test such as immunoassay because of cross-reactivity with an unrelated substance in the urine. For example, fluoroquinolone antibiotics have been reported to cross-react with immunoassay opiate screens [12, 112-114]. Confirmatory tests (GC-MS, LC-MS, etc.), however, are generally highly unlikely to yield false-positive results, although, in some specific cases, particularly clinical ones, false-positives are a possibility. For example, a patient taking Adderall® capsules for attention deficit hyperactivity disorder will get a positive test result for amphetamines, which may be a false-positive for substance abuse [40].

False-negative results (namely, not detecting a drug of abuse when the individual has consumed) can also happen as a result of low levels of the DOAs in urine. There are many ways for (ab)users to tamper testing. These include substitution, *in vivo* adulteration, *in vitro* adulteration and dilution of the urine sample [16, 37, 115].

Urine substitution is achieved through the replacement of a urine specimen with drug-free urine or commercially available synthetic urine that possesses the same essential characteristics as human urine, using devices such as the Butt Wedge™, the Urinator™ and the Whizzinator™. These devices can be easily purchased on the Internet. In extreme cases, catheterization has also been reported for this purpose.

*In vivo* adulteration involves the deliberate intake of a copious volume of water or other fluids to dilute the urine or the intentional ingestion of products such as diuretics designed to increase the metabolism and/or excretion of DOAs to obtain a test result below the cut-off values established. Urine dilution can also be performed by the addition of water directly to the specimen.

*In vitro* adulteration is the act of adding foreign chemicals to a urine sample to interfere with urine drug testing. These include household chemicals, such as over-the-counter eye drops containing tetrahydrozoline, bleach, vinegar, soap, ammonia, drain cleaner, table salt and a variety of commercial products readily available online, such as UrinAid (glutaraldehyde), Klear® (potassium nitrite), Urine Luck (pyridinium chlorochromate) and Stealth® (containing peroxide and peroxidase), many of which are oxidants.

Because more and more individuals who misuse DOAs are motivated to tamper their urine when it comes to drug testing to avoid consequences, such as incarceration, fines, end of employment or suspension from competition in elite sports, significant efforts have been made in developing countermeasures. These include appropriate collection techniques (see earlier discussion) and tests of specimen integrity (appearance, temperature, creatinine, specific gravity, pH and oxidants). Table 9 gives a brief summary of the most relevant findings that suggest adulterated, diluted, substituted or invalid urine. Adulteration with diuretics can be identified by monitoring diuretics in urine specimens, as implemented in anti-doping sport drug testing programs.

**Table 9.** Laboratory findings that suggest urine manipulation [12, 39, 40].

| |
|---|
| **General** |
| • Temperature between 32.2ºC and 37.7ºC |
| • pH between 4.5 and 8.5 |
| **Adulterated** |
| • pH of less than 3 |
| • pH of 11 or greater |
| • Nitrite concentration of 500 μg/mL or greater |
| • Chromium (VI) is present |
| • A halogen (e.g., iodine, fluoride) is present |
| • Glutaraldehyde is present |
| • Pyridine is present |
| • A surfactant is present |

**Table 9.** (Continued).

| |
|---|
| **Diluted** |
| • Creatinine concentration is greater than 5 mg/dL and less than 20 mg/dL |
| • Specific gravity is greater than 1.0010 and less than 1.0030 |
| **Substituted** |
| • Creatinine concentration is less than 2 mg/dL |
| • Specific gravity is less than or equal 1.0010 or greater than or equal to 1.0200 |
| **Invalid** |
| • Creatinine concentration of less than 2 mg/dL and specific gravity of greater than 1.0010 and less than 1.0200 |
| • Creatinine concentration is 2 mg/dL or greater and specific gravity is 1.0010 or less |
| • pH of 3 or greater and less than 4.5 |
| • pH of 9 or greater and less than 11 |
| • Nitrite is 200 μg/mL or greater and less than 500 μg/mL |

Further detailed discussion regarding identification and management of both false-positive and false-negative results in urine drug testing has recently been reported in an excellent SAMHSA manual [40].

Moreover, as is well known, once ingested, DOAs are metabolized into the body. As a result, drug consumption can be proven through the detection of the metabolites, which are indicative of ingestion of a drug, thereby minimizing the possible false-positive results. Major metabolites of the most common DOAs in urine are listed in Table 10.

**Table 10.** Major metabolites of DOAs in urine [10].

| *Drug* | *Major metabolite* |
|---|---|
| Amphetamine | Uncharged drug |
| Methamphetamine | Amphetamine |
| *Barbiturates* | |
| Secobarbital | 3-Hydroxy-secobarbital |
| Pentobarbital | 3-Hydroxy-pentobarbital |
| Amobarbital | 3-Hydroxy-amobarbital |
| Phenobarbital | *p*-Hydroxy-phenobarbital [a] |
| *Benzodiazepines* | |
| Alprazolam | 4-Hydroxy-alprazolam, α-hydroxy-alprazolam |
| Diazepam | Oxazepam [a], nor-diazepam |
| Lorazepam | Conjugated with glucuronic acid |
| Clonazepam | 7-Aminoclonazepam |
| Triazolam | 4-Hydroxy-triazola, α-hydroxy-triazolam |

**Table 10.** (Continued).

| *Drug* | *Major metabolite* |
|---|---|
| Cocaine | Benzoylecgonine, ecgonine methyl ester, nor-cocaine |
| *Opiates (natural and synthetic)* | |
| Heroin | 6-Acetylmorphine, morphine [a] |
| Codeine | Morphine, morphine-3-glucuronide |
| Morphine | Morphine-3-glucuronide |
| Hydrocodone | Hydromorphone |
| Oxycodone | Oxymorphone |
| Methadone | EDDP, EMDP |
| Phencyclidine | Cis- and trans-1-(1-phenyl-4-hydroxycyclohexyl)piperidine |
| Propoxyphene | Nor-propoxyphene |
| Tetrahydrocannabinol | THC-COOH [a] |

[a] Also excreted in urine as a conjugate of glucuronic acid.

UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES
Tatiana Baciu

## 1.3. CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF DRUGS OF ABUSE

UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
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As introduced in earlier sections, in addition to LC and GC, CE is a well-established analytical separation technique that has been successfully applied to determine a broad range of DOAs in different types of matrices, providing the benefits of high efficiency and resolution, possibility of automation, minimal consumption of reagents and low sample volume for analysis, as well as low-cost [46, 78, 100]. However, in spite of these great benefits, it is well known that CE suffers from poor concentration sensitivity, especially when UV detection is employed. To overcome this problem, the coupling of preconcentration strategies to CE has developed into a significant field of research for the detection of many drugs at low concentration levels.

Recently the journal *Trends in Analytical Chemistry* reported our review paper related to the determination of a range of commonly-encountered DOAs and their metabolites in different types of matrices by means of CE and related techniques. In this work, the different electrophoretic methods published between 2007 and 2015 were compared in terms of separation efficiency, sensitivity, time of analysis and potential for automation. The most recently introduced sample preparation and preconcentration techniques were also addressed, as well as a critical discussion of the main strengths and weaknesses of all of these approaches by means of relevant applications. A copy of this manuscript is attached in Section 1.3.1. of the present Doctoral Thesis.

Furthermore, in recent years, other research articles have appeared in the scientific literature dealing with the use of CE-based methodologies for the analysis of several DOAs {Table 11 [116-121]}, which have not been included in our review and, therefore, will be summarized below.

**Table 11**. CE methodologies for determination of DOAs.

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| Twenty-four designer cathinones and phenethylamines | *CZE-UV*<br>Dynamically coated capillary: 64.5 cm × 50 μm i.d.; BGE: CElixir Reagent B (pH 2.5) + 80 mM HP-β-CD; separation voltage: 30 kV; temperature: 15ºC; UV detector at 205 nm | – | Seized samples | Dissolve in BGE | [116] |

**Table 11**. (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| MP, AP, phenethylamine, mephentermine, methoxyphen-amine | *CZE-ESI-MS/MS (QqQ)* Fused-silica capillary: 100 cm × 50 µm i.d.; BGE: 20 mM ammonium formate (pH 2.5); separation voltage: 20 kV; temperature: 25ºC | 2-5 nM [a] | Urine | SDME | [117] |
| KET, NKET | *FASI-CZE-UV* Fused-silica capillary: 45 cm × 50 µm i.d.; BGE: 15 mM Tris phosphate (pH 2.5) + 0.1% HS-γ-CD (w/v); separation voltage: 20 kV; temperature: 20ºC; UV detector at 200 nm | 0.08 ng/mg [b] | Hair | 50 mg; Washing with SDS, water, MeOH; Digestion with 0.1 M HCl (overnight at 37ºC) and LLE with hexane/EtAc (50: 50, v/v); Evapora-tion/reconstitution | [118] |
| 6-AM, CET, COC, heroin, MP, MDEA, MDMA, MOR, mephedrone | *MSS-CZE-UV* Fused-silica capillary: 60 cm × 50 µm i.d.; BGE: 50 mM phosphate buffer (pH 2.5) + 30% v/v ACN; micellar solution: 20 mM phosphate buffer (pH 2.5) + 12 mM SDS; separation voltage: 20 kV; temperature: 20ºC; UV detector at 200 nm; | 15-75 ng/mL [b] | Urine | Centrifugation at 12,000 rpm for 15 min and the supernatant mixed with the micellar solution | [119] |
| THC, THC-OH, THC-COOH | *LVSI-ASEI-sweeping-MEKC-UV* Fused-silica capillary: 40 cm × 50 µm i.d.; BGE: 30 mM phosphate buffer (pH 2.5) + 40% v/v MeOH + 100 mM SDS; sweeping buffer: 200 mM phosphate (pH 2.5); separation voltage: -15 kV; temperature: 25ºC; UV detector at 214 nm | 0.5-10.0 ng/mL [a] | Urine | Hydrolysis with NaOH and LLE with hexane/EtAc (8:2, v/v); Evapora-tion/reconstitution | [120] |

**Table 11**. (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| MP, AP | *CSEI-sweeping-MEKC-UV* Fused-silica capillary: 64.5 cm × 50 μm i.d.; BGE: 100 mM phosphate (pH 2.7) + 20% (v/v) MeOH + 20% (w/v) HS-γ-CD + 20 mM SDS; separation voltage: -18 kV; temperature: 25°C; UV detection at 195 nm | 0.07-0.09 ng/mL [a] | Hair | 3 mg; Incubation in 1 M NaOH (30 min, 70ºC) and LLE with cyclohexane; Evaporation/ reconstitution | [121] |

See Appendix I for the key to abbreviations.
[a] Standard samples.
[b] Real samples.

As can be seen from Table 11, the procedures published used capillary zone electrophoresis (CZE) [116-119] and micellar electrokinetic chromatography (MEKC) [120, 121] with UV [116, 118-121] and MS [117] detection for the screening and/or determination of new psychoactive substances [116], anaesthetics [118], stimulants and opium-related compounds [117, 119, 121], and cannabinoids [120] in urine [117, 119, 120], hair [118, 121] and seized samples [116].

To preconcentrate the analytes contained in the urine, a preconcentration strategy known as micelle to solvent stacking was successfully evaluated, which consists of mixing the sample with a phosphate buffer containing sodium dodecyl sulphate before being injected into the CE instrument [119]. Under the optimized conditions, enrichment factors of up to 55-fold were obtained with UV detection compared to conventional CE, resulting in LOQs of 30-100 ng/mL. In another study, a combination of three on-line stacking strategies, large volume sample injection with anion-selective exhaustive injection and with sweeping, has been established for the same purpose [120]. An improvement in sensitivity of up to 2,000-fold compared to MEKC without electrophoretic preconcentration with LODs (0.5-10.0 ng/mL) below the cut-off levels in urine (listed in Table 4) was obtained.

In the case of hair [118], in order to achieve a suitable sensitivity, a field-amplified sample stacking technique was included, providing an LOQ of 0.25 ng/mg for ketamine and its major metabolite, norketamine. Another highly sensitive method based on the combination of cation-selective exhaustive injection with sweeping in

MEKC for determining amphetamine and methamphetamine in hair samples was recently published, providing more than 10,000-fold sensitivity increase compared to normal MEKC [121]. This strategy allowed quantification of the drugs down to 0.2 ng/mg in only 3 mg of hair, which is in line with the cut-off levels in hair {0.2 ng/mg [122]}.

High enrichment factors may also be achieved by including a clean-up and preconcentration process in the sample preparation step. In this respect, single drop microextraction (SDME) coupled in-line with CE-UV is a suitable and powerful preconcentration and sample clean-up strategy [123]. In in-line SDME-CE-UV, analytes are extracted from the sample solution into an acceptor drop hanging at the inlet tip of the capillary. The enriched drop is then introduced into the capillary for CE analysis. To form the drop at the inlet tip of the capillary, a pressure, which has been carefully optimized in advance, is applied to the outlet vial containing the acceptor phase by programming this step in a commercial CE instrument. In contrast, in-line coupling of SDME and CE-MS has been difficult so far due to the lack of forming an acceptor drop since there is no outlet vial in a conventional CE-ESI-MS configuration.

To overcome this issue, Kim *et al.* [117] used a temporary outlet reservoir containing the run buffer for CE at the ESI tip during the drop formation and extraction. Figure 13 shows a diagram of the procedure followed by the authors for the in-line SDME-CE-ESI-MS/MS. As can be observed in the figure, in-line SDME-CE procedures do not involve any manipulation of the sample by the user, facilitating automatization and miniaturization of CE methodologies. Five basic DOAs, namely methamphetamine, amphetamine, phenethylamine, methoxyphenamine and mephentermine, were extracted from a basic sample solution to an acidic acceptor drop covered with a thin octanol layer. Using this strategy, the sensitivity enhancement for standard samples was up to 130-150-fold compared to CE-MS/MS without the in-line preconcentration. For urine, 30-45-fold sample enrichment was obtained in only 3 min extraction without using any other technique to clean-up the matrix from interfering contaminants.

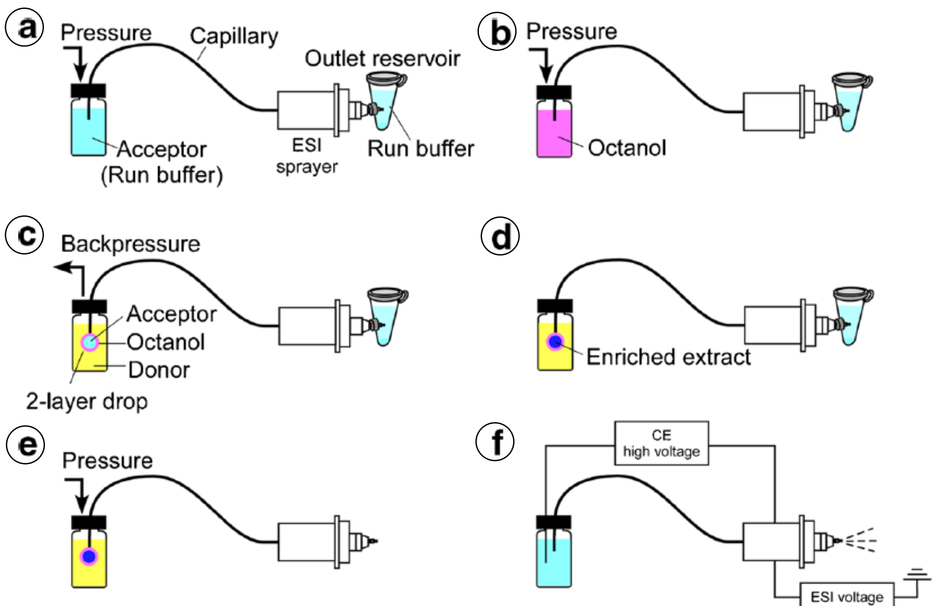


**Figure 13.** Procedures for in-line SDME-CE-MS/MS: acceptor (run buffer) injection into the whole capillary (a), octanol injection (b), two-layer drop formation at the capillary tip by backpressure (vacuum) (c), extraction (d), enriched sample injection from the droplet into the capillary and removal of the outlet reservoir at the ESI tip (e), operation of CE-MS/MS (f). Reproduced from [117] with the permission of Springer-Verlag Berlin Heidelberg, copyright 2012.

The following section includes our review article titled "Capillary electrophoresis and related techniques in the determination of drugs of abuse and their metabolites" published in the journal *Trends in Analytical Chemistry*.

UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES
Tatiana Baciu

### *1.3.1. Capillary electrophoresis and related techniques in the determination of drugs of abuse and their metabolites*

# CAPILLARY ELECTROPHORESIS AND RELATED TECNIQUES IN THE DETERMINATION OF DRUGS OF ABUSE AND THEIR METABOLITES

**Tatiana Baciu, Igor Botello, Francesc Borrull, Marta Calull* and Carme Aguilar**

Department of Analytical Chemistry and Organic Chemistry, Rovira i Virgili University, Campus Sescelades, Marcel·lí Domingo, 1, 43007 Tarragona, Spain

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**Abstract**

Nowadays, so-called drugs of abuse (DOAs) are widely used and pose an undeniable problem at a social level. These compounds have incalculable consequences for society, such as the cost of medical treatment, higher incidence of criminality and economic repercussions. Innovative, modern separation and determination techniques are therefore required for analysis of DOAs. In this work, we review methods based on capillary electrophoresis applied to the determination of illicit drugs and new psychoactive substances in different kinds of matrix, published between 2007 and 2015. We critically discuss the main strengths and weaknesses of all of these approaches by means of relevant applications in clinical practice, forensics, pharmacokinetics, metabolic monitoring and environmental analyses.

***Keywords***: *bioanalysis/capillary electrophoresis/determination of drugs of abuse/illicit drug/ metabolite/monitoring/psychoactive substance/sample preparation/separation*

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## 1. Introduction

Drugs of abuse (DOAs) is a term given to drugs used excessively on a persistent or sporadic basis inconsistent with or unrelated to acceptable medical practice, the abuse of which can generally lead to physical and mental damage, and, in some cases, to dependence and addiction [1]. Consequently, there is a need for the continuous development of methods for the efficient determination of DOAs and their metabolites in biological samples. The matrices most commonly used for this purpose are urine, saliva, hair, blood and sweat, while breath is most commonly used for alcohol testing. Each matrix has particular strengths and weaknesses, which have already been discussed elsewhere [2, 3]. The choice of sample is influenced by sample collection procedures, costs, windows of drug detection and the degree of incorporation of drugs and their metabolites into the biological matrix, among other factors. However, as a consequence of their use, these compounds can reach wastewater-treatment plants and can eventually be released to environmental waters [4]. As a result, DOAs have been found in different surface waters at ng/L-concentration levels [4, 5].

Identification and determination of the broad range of DOAs and their metabolites in different kinds of matrix has become a challenging task for analytical chemists. In this context, organizations such as European Workplace Drug Testing Society, Society of Hair Testing and Substance Abuse, and Mental Health Services Administration, have established cut-off concentrations, above which any analytical finding indicates positive for drug use in order to avoid false-positive results. Analytical methods must therefore provide accurate, reliable results from the test sample. In this regard, several analytical procedures have been reported in the scientific literature, most of which are based on gas chromatography (GC) or liquid chromatography (LC) [6–8]. Besides these techniques, capillary electrophoresis (CE) and related techniques, such as micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC) and capillary electrochromatography (CEC), have also been successfully applied for this purpose, shown by the large number of applications developed [8–11]. This increasing interest in CE-based techniques as analytical tools is certainly based on their high efficiency, high resolution power, low

reagent and sample consumption, automation and the fact that they represent low cost candidates, compared to other chromatographic techniques.

However, the widespread use of chromatography-based methodologies is due to limitations that CE-based methodologies display in relation to their relative lack of sensitivity. The development of strategies that increase this sensitivity has therefore become an important issue and numerous approaches have been published [12–14].

The goal of this review is to provide an overview of the state of the art and the latest trends of the CE-based methods proposed since 2007 to the present for the determination of DOAs and their metabolites. Recently, Cruces-Blanco *et al.* [15] reviewed the use of CE and related techniques for the analysis of DOAs and their metabolites in biological specimens of interest in forensic toxicology, particularly blood, urine and hair samples, focusing on some sample preconcentration methods to enhance sensitivity and the use of different detection modes.

Our review provides the most relevant improvements and innovations in the CE-based methodologies for the analysis of a range of commonly encountered illicit substances in different types of matrix, such as seized materials, and biological and environmental samples. We compare the different electrophoretic methods published during the period covered by this review in terms of separation efficiency, sensitivity and time of analysis and/or potential for automation. We also address the most recently introduced sample preparation and preconcentration techniques. We critically discuss the main strengths and weaknesses of all of these approaches by means of relevant applications.

We divide the article into drug classes with comprehensive, up-to-date tables, providing the reader with more detailed information related to the new trends in the analysis of a specific group of compounds in which she/he can be interested by means of CE-based methodologies. We selected stimulants, such as amphetamine-type stimulants (ATSs) and cocaine, depressants, such as barbiturates and benzodiazepines (BZDs), opium-related compounds, cannabinoids and new psychoactive substances as the target drugs because they are the most frequently abused, illicit drugs.

## 2. Stimulants

Stimulants are a class of psychoactive substances that increase activity

in the brain, causing temporary changes in alertness, mood and awareness. These drugs were historically used for treatment (e.g., respiratory problems, obesity, and neurological disorders), but, as their potential for abuse and addiction became apparent, medical use of stimulants began to decrease. Nowadays, stimulants are prescribed to treat only a few health conditions, such as attention deficit hyperactivity disorders (ADHDs), narcolepsy and, occasionally, depression. The most commonly used street drugs that fall into this category are ATSs and cocaine [16]. With this in mind, this section consists of two parts, the first focusing on ATSs and the second on cocaine, with the CE-based analytical methods currently used being reviewed and summarized in Tables 1 [17–33] and 2 [34–44], respectively.

In view of the tendency for polydrug consumption, multi-analyte methods have also been reported, including those with different chemical and physical properties. In this respect, Table 1 shows analytical methods that determine ATSs together with other drugs, while Table 2 shows methods designed to detect cocaine together with other drugs.

*2.1. Amphetamine-type stimulants*

ATSs are a group of drugs mainly including amphetamine and methamphetamine. Besides these drugs, other substances also fall into this group, such as fenethylline, ephedrine, pseudoephedrine, methylphenidate and 3,4−methylenedioxy-methamphetamine. The abuse of ATSs is global and a growing phenomenon, and, recently, there was an increase in the production and abuse of ATSs worldwide [16], so analytical methods for their efficient, accurate determination are needed.

These illicit substances have been widely studied by CE, with capillary zone electrophoresis (CZE) [17–24, 27–31] being the most commonly used separation mode, although MEKC [25, 26, 32] and MEEKC [33] have also been reported as approaches for separating these compounds. The most extensively used detector is ultraviolet (UV) but other detectors, such as electrochemical (EC) [17], laser-induced fluorescence (LIF) [18], mass spectrometry (MS) [19] and capacitively coupled contactless conductivity ($C^4D$) [20, 21], have also been employed for this purpose.

With respect to the samples analyzed, the existing literature mainly focuses on biological samples,

**Table 1.** Overview list of capillary electrophoresis (CE) methodologies for determination of amphetamine-type stimulants (ATSs).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| MP, MDA, MDMA | *CZE-EC/ECL* Fused-silica capillary: 50 cm × 25 µm i.d., BGE: 50 mM phosphate buffer, detection buffer: 100 mM phosphate buffer (pH 9.0) + 5 mM $[Ru(bpy)_3]Cl_2$, separation voltage: 25 kV, temperature: 25ºC | 1.6 $10^{-7}$-3.3 $10^{-8}$ mol/L [a] | Urine | LLE with EtAc | [17] |
| PE, EP, AP, MP, MDA, MDMA | *CZE-LIF* Fused-silica capillary: 50 cm × 75 µm i.d., BGE: 20 mM borate buffer + NaOH (pH 12.0), separation voltage: 25 kV, temperature: 25ºC | 0.2 ng/mL [b] | Blood and urine | SPE with Oasis HLB cartridge | [18] |
| DOM, DOET, DOPR | *CZE-MS* Fused-silica capillary: 120 cm × 50 µm i.d., BGE: 10 mM phosphate buffer (pH 4.5), separation voltage: 10 kV, temperature: 25ºC | 3.9-4.6 ng/mL [b] | Urine | SPE with Bond Elut $C_{18}$ cartridge | [19] |
| 2-4-MPEA, MBA, PEA, 2-MMA, AM-C-BD, MPEA, MMBA, AP, DexAP, MP | *CZE-C⁴D-UV* Fused-silica capillary: 90 cm × 50 µm i.d., BGE: 25 mM sodium acetate + 75 mM AA (pH 4.55) + 30 mM HP-β-CD, separation voltage: 30 kV, temperature: 25ºC, UV detection at 200 nm | 1,300 ng/mL [a] | Street-grade tablets | – | [20] |
| AP, MP, EP, PE, NE, NPE, | *CZE-C⁴D* Fused-silica capillary: 50 cm × 50 µm i.d., BGE: 500 mM AA (pH 2.6) + 5 mM CM-β-CD + 5 mM 18-C-6-TA, separation voltage: 15 kV, temperature: 25ºC | – | Urine | LLE with EtAc | [21] |

**Table 1.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| MP, AP DMA, β-HMP, EP, NE, MDA, MEPH, MDMA, MDEA | *CZE-UV* Poly(vinyl alcohol)-coated capillary (home-made) and chemically modified capillary with diol groups (commercial): 64.5 cm × 50 μm i.d., BGE: 125 mM Tris (pH 6.15) + 6 mM heptakis (2,6-di-*O*-methyl)-β-CD + 12 mM β-CD, separation voltage: 30 kV, temperature: 25°C, UV detection at 195 nm | 410-940 ng/mL [a] | Urine | LLE | [22] |
| MP, AP, EP, PE, NE, NPE | *CZE-UV* Chemically modified capillary with sulfate groups (commercial): 32.5 cm × 50 μm i.d., BGE: 50 mM phosphate buffer (pH 4.5) + 10 mM HS-γ-CD, separation voltage: 10 kV, temperature: 15°C, UV detection at 195 nm | – | Drug seizures | – | [23] |
| *Threo*-methyl-phenidate | *FASI-CZE-UV* Fused-silica capillary: 40 cm × 50 μm i.d., BGE: 50 mM phosphate buffer (pH 3.0) + 30 mM TEA + 20 mM HP-β-CD, separation voltage: 20 kV, temperature: 25°C, UV detection at 200 nm | LOQ: 0.5 ng/mL [b] | Saliva | LLE with hexane | [24] |
| AP, MP, MDMA | *CSEI-Sweeping-MEKC-UV* Fused-silica capillary: 60 cm × 75 μm i.d., BGE: 100 mM phosphate buffer (pH 3.0) + 20% MeOH + 20 mM SDS, separation voltage: 18 kV, temperature: 25°C, UV detection at 200 nm | – | – | – | [25] |

**Table 1.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| MP, KET, MOR, COD | *CSEI-sweeping-MEKC-UV* Fused-silica capillary: 40 cm × 50 µm i.d., BGE: 25 mM phosphate buffer (pH 2.5) + 20% MeOH + 100 mM SDS, separation voltage: -20 kV, temperature: 25ºC, UV detection at 200 nm | 0.006-0.008 ng/mg [b] | Hair | Digestion with 100 mM HCl (2 h at room temperature) and LLE with EtAc | [26] |
| MDMA, LSD, PCP | FASI-CZE-UV Fused-silica capillary: 72 cm × 50 µm i.d., BGE: 100 mM phosphate buffer (pH 6.0), separation voltage: 25 kV, temperature: 20ºC, UV detection at 205 nm | 1.00-4.52 ng/mL [b] | Urine | DLLME | [27] |
| AP, KET, MP, MDA, MDMA | *FASI-CZE-UV* Fused-silica capillary: 60 cm × 75 µm i.d., BGE: 30 mM phosphate buffer (pH 2.0) + 15% v/v ACN, separation voltage: 20 kV, temperature: 25ºC, UV detection at 214 nm | 4.0-6.0 ng/mL [b] | Urine | MSPE with $Fe_3O_4$/silica/poly (methacrylic acid-*co*-vinylbenzyl chloride-*co*-divinyl-benzene) magnetic microspheres | [28] |
| AP, KET, MP, MDA, MDMA, 6-AC, COD, heroin | *FASI-CZE-UV* Fused-silica capillary: 60 cm × 75 µm i.d., BGE: 30 mM phosphate buffer (pH 2.0) + 15% v/v ACN, separation voltage: 20 kV, temperature: 25ºC, UV detection at 214 nm | 15-105 ng/mL [b] | Urine | MSPE with $Fe_3O_4$/silica/poly (methacrylic acid-*co*-ethylene glycol dimethacrylate) magnetic microspheres | [29] |
| AP, PCA, 2-amino-1,2-diphenyl-ethanol | *CZE-UV* Fused-silica capillary: 60 cm × 50 µm i.d., BGE: 50 mM bis (2-hydroxyethyl) imino tris (hydroxymethyl) methane (pH 4.0) + 0.8 mM 18-C-6-TA, separation voltage: 25 kV, temperature: 25ºC, UV detection at 200 nm | 0.4-2.0 ng/mL [b] | Urine | SDME | [30] |

**Table 1.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| Heroin, MP, MDMA, KET | *CZE-UV* Fused-silica capillary: 37 cm × 50 μm i.d., BGE: 100 mM phosphate buffer (pH 3.23) + 20 mM β-CD, separation voltage: 20 kV, temperature: 25ºC, UV detection at 200 nm | 0.05-0.20 ng/mL [a] | Bank-notes, kraft paper, plastic bag, silver paper | DLLME | [31] |
| EP, NE, PE | *MEKC-UV* Fused-silica capillary: 64.5 cm × 75 μm i.d., BGE: 25 mM borate buffer (pH 9.3) + 1.0 mM SDS, separation voltage: 15 kV, temperature: 25ºC, UV detection at 194 nm | 12-33 ng/mL [b] | Urine | DLLME or USAEME | [32] |
| Caffeine, theophylline, barbital, phenobarbital, MOR, 6-AM, 3-AM, COD, EP, heroin, AP, 6-AC, MP, thebaine, papaverine, narcotine | *MEEKC-UV* Fused-silica capillary: 40 cm x 75 μm i.d., BGE: 89.8% v/v 5 mM borate buffer (pH 9.5) + 3.3% v/v SDS + 6.0% v/v 1-butanol/0.9% octane, separation voltage: 20 kV, temperature: 25ºC, UV detection at 200 nm | − | Sample | − | [33] |

[a] Standard samples.
[b] Real samples.

particularly urine [17−19, 21, 22, 27−30, 32], as well as blood [18], saliva [24] and hair [26]. However, there are other types of sample, such as street-grade tablets, banknotes, Kraft paper, plastic bags, silver paper and drug seizures, which have also been studied [20, 23, 31]. In the tables in this article, unless otherwise indicated, the term urine refers to human urine.

The use of CE for chiral analysis is also gaining momentum. In this respect, a review of the chiral analysis of ATSs in biological samples was published in 2011, including a

discussion of CE applications [45]. To summarize, the most commonly employed method for this purpose is to include native cyclodextrins (CDs) in the background electrolyte (BGE), with β-cyclodextrin (β-CD) [45] being the most widely used, although crown ethers, such as 18-crown-6-tetracarboxylic acid [21, 30], have also been used. Chemically modified CDs, especially the sulfated derivatives of β-CD [i.e., sulfated-β-CD, hydroxy-propyl-β-CD, heptakis-(2,6-*O*-dimethyl)-β-CD and heptakis-(2,3,6-*O*-trimethyl)-β-CD], have also been shown to provide good enantio-selectivity [20–22, 24].

Recently, a research paper was published dealing with the optimization of a fast separation technique (8 min) for six enantiomeric pairs of ATSs using highly sulfated-γ-CD as the chiral selector [23]. To improve control of the electroosmotic flow and to avoid the consequent shift in analyte peaks, and to improve the reproducibility of migration times for analytes by CE, four types of commercial chemically modified capillaries were evaluated, namely, FunCap-CE/Type D (possessing diol groups), Type A (amino groups), Type C (carboxyl groups) and Type S (sulfate groups). The Type S capillary gave better results in terms of reproducibility (standard deviations ≤0.05), proving to be an excellent tool for rapid routine screening of these illicit drugs, for example, in illegal drug laboratories and in drug seizures.

As mentioned, CE represents an attractive alternative to chromato-graphic approaches because only a few nL of sample need to be injected. However, it is well known that this advantage leads to relatively poor concentration limits of detection (LODs), and this is a problem, as many analytes are present at very low concentrations. In order to deal with the small amount of analytes injected in CE, various approaches have been developed [12, 14], mostly using preconcentration procedures, such as sweeping [25, 26] and field amplified sample injection (FASI) [24, 27–29]. For example, Airado-Rodríguez *et al.* [27] developed a CZE method with UV detection for the analysis of 3, 4−methylenedioxymethamphetamine, lysergic acid diethylamide and phencyclidine in urine. With the use of 100 mM phosphate at pH 6.0 as the BGE and FASI for on-line sample preconcentration, LODs of 1.0–4.5 ng/mL were reached. The method was successfully applied to the analysis of the three drugs in the abusers' urine, proving to be a promising strategy that should be further applied to assess other DOAs, since there is a common tendency towards polydrug consumption.

A combination of two on-line stacking strategies, cation selective exhaustive injection (CSEI) with sweeping, with MEKC-UV, was established for the analysis of methamphetamine and other DOAs in hair by Lin *et al.* [26]. Using this strategy, the sensitivity enhancement was about 1,000-fold compared to MEKC separation without electrophoretic preconcentration. Hair samples were analyzed using liquid-liquid extraction (LLE) for sample clean-up prior to analysis, reaching LODs (0.05–0.20 ng/mg) that are in accordance with the cut-offs recommended by the Society of Hair Testing (0.2 ng/mg). This method has been shown to be useful for detecting trace amounts of these illicit drugs in the addicts' hair, so one can conclude that electrophoretic preconcentration techniques are quite suitable for sensitivity improvement in the toxicological analysis of complex matrices, such as human hair.

Moreover, high sensitivity may also be obtained by including a clean-up and preconcentration process in the sample preparation step. To date, classical techniques, such as LLE [17, 21, 22, 24, 26] and solid-phase extraction (SPE) [18, 19], have often been applied for this purpose, although, recently, magnetic SPE (MSPE), a novel mode of SPE based on the use of magnetic adsorbents, emerged as a powerful technique [28, 29]. In this respect, an MSPE procedure combined with FASI-CZE-UV for the determination of ATSs and ketamine in urine was presented by Gao *et al.* [28]. Using magnetite/silica/ poly(methacrylic acid-*co*-vinylbenzyl chloride-*co*-divinylbenzene) magnetic microspheres, the extraction was completed within 3 min with recoveries in the range 84.0–123%. The proposed method was successfully applied to the analysis of abusers' urine with LODs in the range 4.0–6.0 ng/mL. However, these MSPE particles are not yet commercially available, and this may be considered a drawback of this approach, since the reproducibility of this method may be affected.

In recent years, greener pretreatment techniques were proposed, as they involve a reduction of organic solvent volumes with the subsequent production of less toxic residues. In this respect, CE-based methods have been reported for ATSs determination in different matrices using single-drop micro-extraction (SDME) [30], dispersive liquid-liquid microextraction (DLLME) [27, 31, 32] or ultrasound assisted emulsification microextraction (USAEME) [32] for cleaning up the sample and preconcentration of the analytes.

For example, Choi *et al.* [30] successfully employed a rapid, efficient preconcentration strategy based on the use of SDME in a three phase design coupled in-line to CZE for the chiral analysis of three primary amine compounds, including amphetamine, in urine. The analytes were first extracted from the alkalized urine sample into a drop of *n*-octanol layered over the urine sample and then into a microdrop of the acceptor phase (120 mM bis-tris methane/citric acid at pH 4.0) suspended at the capillary inlet tip. The enriched acceptor phase was directly injected into the capillary and then analyzed in a BGE composed of 50 mM bis-tris methane/citric acid with 0.8 mM 18-crown-6-tetracarboxylic acid at pH 4.0. The method achieved LODs of 0.4–2.0 ng/mL with a 1,000-fold increase in UV-detection sensitivity. Its application to urine was shown through the analysis of some blank samples spiked with the analytes and no validation assay was carried out. Nevertheless, the proposed in-line SDME procedure allows commercial CE instruments to handle complex matrices directly, facilitating automation and miniaturization of CE methodologies.

Recently, the analytical performance of DLLME and USAEME were compared for the extraction of three ephedrines from urine prior to their determination by FASI-MEKC [32]. Under optimal extraction and stacking conditions, enrichment factors of up to 140 and 1,750 were obtained compared to conventional CZE, resulting in LODs of 12–33 ng/mL and 1.0–2.8 ng/mL with DLLME/FASI-MEKC and USAEME/FASI-MEKC, respectively. With satisfactory recoveries (94.5–103.3%) and good values of intra-day relative standard deviations (RSDs) (≤8.2%), both methods would be appropriate as a routine tool for clean-up and sensitivity enhancement in the determination of substances of abuse, due to their simplicity, effectiveness, low cost and environmental friendliness.

### *2.2. Cocaine*

Cocaine, an alkaloid derived from the *Erythroxylum coca* plant, is known to be a powerful addictive stimulant that directly affects the brain. For hundreds of years, it has been used as a local anesthetic during surgeries. However, in recent years, cocaine abuse became epidemic. Once ingested, it is primarily hydrolyzed in the body to benzoylecgonine and ecgonine methyl ester, but it is also metabolized to norcocaine and other metabolites. As a result, cocaine abuse can be proved through detection of its metabolites, which are

indicative of ingestion of the drug [16].

In 2010, Janicka *et al.* [46] reviewed analytical protocols proposed within the past 10 years for analysis of cocaine and its metabolites in biological samples, including CE-based methods. With this in mind, this section focuses on papers published since 2010 up to the present related to the determination of these compounds by CE in biological samples, as well as in other matrices, such as environmental samples and banknotes, as these have also been analyzed for this purpose.

As shown in Table 2, procedures using different CE modes have been reported, such as CZE [34–41], MEKC [42] and CEC [43, 44] with MS [38–41, 43] and UV [34–37, 42, 44] for determination of several DOAs, including cocaine and its metabolites.

As is well known, paper money can become contaminated with cocaine during drug deals and/or directly through drug use with users snorting the drug through rolled bills. In this context, a fast CZE method with UV detection was proposed for the determination cocaine on Brazilian banknotes [34]. Cocaine was extracted from banknotes by sonication for 8 min with acetonitrile and then directly loaded into the CZE-UV instrument without a concentration procedure, achieving a limit of quantification (LOQ) of 800 ng/mL. The method developed was successfully applied to determine cocaine on banknotes obtained directly from general circulation, proving to be a useful tool for identifying the presence of this drug, for example, in forensic criminology analysis of this type of sample.

Some authors have developed strategies to increase the sensitivity of CE for the determination of cocaine and its metabolites in environmental samples [36], as well as biological samples [35, 37–44], using different preconcentration techniques, such as in-line SPE [36, 37], FASI [38, 39], pH-mediated stacking [40], DLLME [41], sweeping [42] and SDME [44].

Botello *et al.* [36] described the use of in-line SPE, as a clean-up step and enrichment technique, in combination with CZE for determining cocaine, 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine, codeine and 6-acetylmorphine in environmental waters. The SPE-CE device consisted of a short length of capillary of 2 mm filled with a hydrophilic-lipophilic-balance sorbent and inserted near to the inlet end of the separation capillary. With UV detection and simple filtration of the urine samples prior to in-line SPE-CE analysis, LODs of 0.07–0.27 ng/mL were achieved. Compared with CZE

**Table 2.** Overview list of capillary electrophoresis (CE) methodologies for determination of cocaine.

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| COC | *CZE-UV* Fused-silica capillary: 48.5 cm × 75 µm i.d., BGE: 60 mM Tris + 20 mM 2-hydroxyisobutyric acid (pH 8.4), separation voltage: 25 kV, temperature: 20ºC, UV detection at 200 nm | 200 ng/mL [a] | Bank-notes | Ultrasound extraction with ACN | [34] |
| AP, MP, 6-AM, MDA, MDMA, MDEA, KET, COC, COE, lidocaine, MOR, heroin | *CZE-UV* Fused-silica capillary: 48.5 cm × 75 µm i.d., BGE: 95% v/v 20 mM Tris + 0.4% TEA (pH 2.5), separation voltage: 25 kV, temperature: 20ºC, UV detection at 195 and 208 nm | 1.0-5.0 ng/mL [a] | Vitreous humour | LLE with EtAc | [35] |
| EDDP, COC, COD, 6-AM | *In-line SPE-CZE-UV* Fused-silica capillary: 65.5 cm × 50 µm i.d., BGE: 80 mM phosphate buffer (pH 3.0), separation voltage: 30 kV, temperature: 25ºC, UV detection at 200 nm | 0.07-0.27 ng/mL [b] | Tap and river water | Filtration | [36] |
| COC, BZE | *In-line SPE-CZE-UV* Fused-silica capillary: 65.5 cm × 50 µm i.d., BGE: 55 mM phosphate buffer (pH 2.55), separation voltage: 30 kV, temperature: 25ºC, UV detection at 200 nm | 0.02-0.10 ng/mg [b] | Hair | Digestion with 100 mM HCl (overnight at 45 ºC) | [37] |
| COC, COE, BZE, NCOC, EME | *FASI-CZE-ESI-MS(IT)* Poly(vinylalcohol)-coated capillary (homemade): 50 cm × 75 µm i.d., BGE: 15 mM ammonium formate (pH 9.5), separation voltage: 25 kV, temperature: 20ºC | 1.5-10 ng/mL [a] | Urine | SPE with Supelclean strong cation exchange cartridge | [38] |

**Table 2.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| MDA, MDMA, MTD, COC, MOR, 6-AM | *FASI-CZE-ESI–MS(TOF)* Fused-silica capillary: 100 cm × 75 μm i.d., BGE: 50 mM ammonium phosphate (pH 6.5), separation voltage: -15 kV, temperature: 25ºC | – | Hair | Digestion with 100 mM HCl (overnight at 45ºC) and LLE with Toxi-Tubes A | [39] |
| MOR, COD, 6-AM, MTD, EMOR, fentanyl, pethidine, buprenorphine, nalbuphine, dextro-methorphan, MTD, EDDP, propoxyphene, AP, MP, MDA, KET, MDMA, MDEA, N-methyl-1-(3,4-methylenedioxy-phenyl)-2-butanamine, EP, PE, NE, methylphenidate, COC, COE, AME, metoprolol, procaine, trimipramine | *pH-mediated stacking-CZE-ESI-MS (TOF)* Fused-silica capillary: 80 cm × 50 μm i.d., BGE: 20 mM ammonium formate (pH 2.5), separation voltage: 30 kV, temperature: 25ºC | 2-200 ng/mL [b] | Urine | Dilution | [40] |
| MOR, COD, 6-AM, MTD EMOR, fentanyl, pethidine, buprenorphine, nalbuphine, | *CZE-ESI-MS(TOF)* Fused-silica capillary: 80 cm × 50 μm i.d., BGE: 20 mM ammonium formate (pH 2.5), separation voltage: 30 kV, temperature: 25ºC | 0.1-10 ng/mL [b] | Urine | DLLME | [41] |

**Table 2.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| dextro-methorphan, MTD, EDDP, propoxyphene, AP, MP, MDA, KET, MDMA, MDEA, N-methyl-1-(3,4-methylenedioxy-phenyl)-2-butanamine, EP, PE, NE, methylphenidate, COC, COE, AME, metoprolol, procaine, trimipramine | | | | | |
| COC, BZE, NCOC, COE | *CSEI-sweeping-MEKC-UV* Fused-silica capillary: 60 cm × 50 μm i.d., BGE: 100 mM phosphoric acid (pH 1.8) + 75 mM SDS + 10% v/v iPrOH + 10% v/v THF, separation voltage: -20 kV, temperature: 25ºC, UV detection at 230 nm | 0.03-0.24 ng/mL [a] | Urine | SPE with Oasis MCX cartridge | [42] |
| AP, MP, MDMA MDA, MDEA, COC, MOR, COD, heroin | *CEC-ESI-MS(IT)* Capillary: 30 cm × 100 μm i.d., packed 3 μm with cyano derivatized silica stationary phase, mobile phase: 25 mM ammonium formate (pH 3.0) + 30% v/v ACN, separation voltage: 12 kV, temperature: 20ºC | 0.78-3.12 ng/mL [a] | Urine | SPE with strong cation exchange cartridge | [43] |

**Table 2.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| EP, COC, strychnine, MOR, caffeine, theophylline, piroxicam | *Open-tubular CEC-UV* MWNTS immobilize fused- silica capillary: 57 cm × 50 µm i.d., mobile phase: 20 mM borate buffer (pH 9.0), separation voltage: 15 kV, temperature: 25ºC, UV detection at 214 nm | 0.94-17.64 ng/mL [a] | Horse urine | SDME | [44] |

[a] Standard samples.

[b] Real samples.

without preconcentration, this method improved sensitivity up to 5,300-fold. With high recoveries (85–97%) and good values of RSDs (<11% in both intra-day and day-to-day experiments), as well as consumption of very low quantities of organic solvent, the developed method has proved to be very environmentally friendly, simple and cheap. Figure 1 shows the electropherogram obtained from the analysis of river water following the proposed in-line SPE-CZE-UV procedure.

Later, the same group developed and validated a method for the simultaneous determination of cocaine and its major metabolite, benzoylecgonine, in hair [37]. An in-line SPE-CZE approach similar to that previously described was applied in conjunction with an extraction process of the analytes based on a digestion procedure in an acidic medium of the hair matrix prior to the in-line SPE-CZE-UV analysis. LOD values were 0.02 ng/mg for cocaine and 0.10 ng/mg for benzoylecgonine, which are reasonably suitable for therapeutic drug monitoring, forensic determinations and doping control. The method was successfully applied to hair samples of cocaine abusers who were undergoing a drug rehabilitation program on the basis of segmental analysis to determine their compliance with the therapy. The major disadvantage of these methods [36, 37] is that the in-line SPE-CE devices are not yet commercially available and this fact may have an influence on the reproducibility of the method.

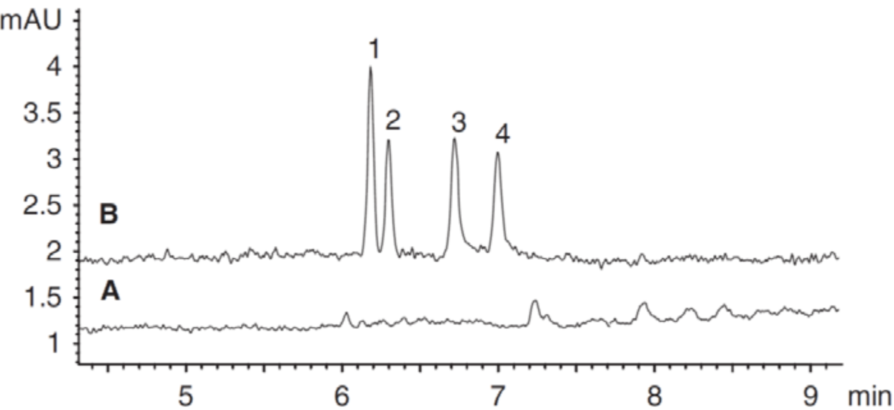


**Figure 1.** (A) Electropherogram of blank river water. (B) Electropherogram of river water spiked with the analytes at a concentration of 1.0 ng/mL analyzed by in-line SPE-CZE-UV. Peak identification: (1) 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, (2) cocaine, (3) codeine and (4) 6-acetylmorphine. Reproduced from [36] with the permission of Elsevier, ©2010.

A simple FASI method was described for the analysis of cocaine and its metabolites in urine by CZE coupled to electrospray ionization (ESI)-ion-trap (IT)MS via a pressurized nano-liquid-junction interface [38]. The use of this device combined with the on-line preconcentration technique increased the detection sensitivity of the method, reaching LODs of 1.5–10.0 ng/mL. The analytical method developed operated under nano flow conditions and provided high sensitivity. However, its application to urine was shown only through the analysis of some blank samples spiked with the analytes and no complete method validation was carried out.

Recently, Kohler *et al.* [40] used pH-mediated stacking in a two step CE-MS method for the analysis of 30 toxicological compounds (amphet-amines and their derivatives, opiates, cocaine and its metabolites, and pharmaceuticals) in urine. The screening step was performed by CZE-ESI-time-of-flight (TOF)MS. pH-mediated stacking was applied with a short plug of 7% w/v ammonium hydroxide injected prior to the urine sample being diluted with BGE and water (1:1:8), reaching LODs as low as 2.0 ng/mL. Compound quantitation for positive samples was performed by CZE-ESI-triple quadrupole (QqQ)-MS/MS, achieving LOQs as low as 10.0 ng/mL in real samples, which are far below the cut-off levels in urine {300 ng/mL [47]}. The same group also presented a different approach using

DLLME as the preconcentration technique with CZE-ESI-(TOF)MS for the toxicological screening of the same illicit substances in urine with LODs at the sub-ng/mL level [41]. Both CZE-ESI-(TOF)MS methods [40, 41] were successfully applied to the analysis of real cases. Compared to other mass spectrometers, such as (IT)MS [38], CZE coupled to (TOF)MS allowed identification of a larger number of different kinds of DOA with better sensitivity, which is very useful in therapeutic drug monitoring, drug rehabilitation programs, doping control and forensic analysis. However, the use of (QqQ)-MS/MS can greatly enhance selectivity using the selected reaction monitoring mode that ensures more accurate measurements and lower LOQs by reducing the chemical noise, providing, in this way, a high level of certainty in identification.

With respect to the use of CEC, Aturki *et al.* [43], who described a method for the determination of nine DOAs, including cocaine, in urine using columns packed with cyano as the stationary phase coupled to ESI-(IT)MS. The coupling was achieved using a liquid-junction interface, providing several advantages, such as high sensitivity (LODs of 0.78–3.12 ng/mL) and short analysis time (13 min), as well as unambiguous identification and confirmation using MS/MS.

Open-tubular CEC has also been proposed for the analysis of illicit drugs, including cocaine, using multi-wall carbon nanotubes (MWCNTs) immobilized into a fused-silica capillary as the stationary phase [44]. Good run-to-run (≤2.83%), day-to-day (≤4.32%) and capillary-to-capillary (≤2.35%) reproducibility have been obtained. Moreover, the regeneration of the functionalized capillary allows its reusability for at least six months. An off-line SDME procedure was used for the preconcentration of the analytes with enrichment factors of 38–102-fold. Both CEC-based methods [43, 44] may be advantageously employed in doping control and forensic analysis.

## 3. Depressants

Depressants, also referred to as sedatives and tranquilizers, are chemicals that can slow brain activity. This property makes them suitable for treating anxiety and sleep disorders. Among the medications commonly prescribed for these purposes are barbiturates and BZDs. Despite their beneficial effects, these drugs have the potential for abuse and should be used only as prescribed [48]. With this in mind, this section consists of two parts, the first focusing on barbiturates and the

**Table 3.** Overview list of capillary electrophoresis (CE) methodologies for determination of depressants.

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| Barbital acid, barbital, phenobarbital, pentobarbital, amobarbital, thiobarbituric acid, N-methyl-5-phenylethyl barbital acid, 5-cyclohexenyl-5-ethyl barbital acid | *CZE-UV* Fused-silica capillary: 42 cm × 50 μm i.d., BGE: 20 mM borate buffer (pH 10.0) + 4% w/v hexadimethrine bromide + 2.06 mM α-CD, separation voltage: -25 kV, temperature: 25ºC, UV detection at 200 nm | 870-3,500 ng/mL [a] | Urine | Filtration | [50] |
| Barbital, amobarbital, phenobarbital, secobarbital | *LVSS-CZE-UV* Fused-silica capillary: 60.5 cm × 75 μm i.d., BGE: 40 mM borate buffer (pH 8) + 20% v/v methanol, separation voltage: 20 kV, temperature: 25ºC, UV detection at 214 nm | 15-57 ng/mL [a] | Plasma | LLE with DCM | [51] |
| Barbital, phenobarbital, secobarbital | *EKS-CZE-UV* Fused-silica capillary: 100 cm × 75 μm i.d., BGE: 20 mM borate buffer (pH 9.15), terminating electrolyte: 100 mM CHES, leading electrolyte: 50 mM sodium chloride, separation voltage: -30 kV, temperature: 25ºC, UV detection at 214 nm | 8-15 ng/mL [b] | Urine | LLE with EtAc/hexane 40:60 v/v | [52] |
| Phenobarbital, p-hydroxy-phenobarbital | *Reverse migrating pseudo-stationary phase-MEEKC-UV* Fused-silica capillary: 50 cm × 75 μm i.d., BGE: 0.8% v/v EtAc + 6.6% v/v butan-2-ol + 1.0% v/v acetonitrile + 2.0% w/v SDS | 16.8 ng/mL [b] | Rat urine | SPE | [53] |

**Table 3.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample treatment* | *Ref.* |
|---|---|---|---|---|---|
| | + 89.6% v/v 7.5 mM ammonium formate (pH 8.0), separation voltage: 25 kV, temperature: 25°C, UV detection at 214 nm | | | | |
| Nitrazepam, oxazepam, alprazolam, flunitrazepam, temazepam, diazepam, 7-AN, 7-AC, 7-AF | *CZE-UV* Poly(diallyldimethylammonium chloride) and dextran sulphate-coated capillary: 69 cm × 50 μm i.d., BGE: 100 mM ammonium phosphate (pH 2.5), separation voltage: 28 kV, temperature: 25°C, UV detection at 200 nm | 2,700-41,500 ng/mL [a] | Beverages | – | [54] |
| Diazepam, clorazepate, chlordiazepoxide, bromazepam, nitrazepam, alprazeolam, flunitrazepam | *MEKC-UV* Fused-silica capillary: 50 cm × 50 μm i.d., BGE: 20 mM phosphate buffer (pH 7.0) + 400 mM lithium bis (trifluoro-methanesulfonyl) imide, separation voltage: 15 kV, temperature: 25°C, UV detection at 230 nm | – | – | – | [55] |
| Lorazepam, bromazepam, clorazepate, nitrazepam, diazepam, alprazolam, flunitrazepam | *MEKC-UV* Fused-silica capillary: 50 cm × 50 μm i.d., BGE: 20 mM phosphate buffer (pH 7.0) + 55% v/v methanol + 170 mM 1-butyl-3- methylimidazolium bis (trifluoromethanesulfonyl) imide + 10 mM SDS, separation voltage: 20 kV, temperature: 25°C, UV detection at 230 nm | 2,740-4,420 ng/mL [a] | Urine | SPE with Oasis mixed-mode cation exchange cartridge | [56] |

**Table 3.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| Alprazolam, bromazepam, medazepam, nitrazepam, chlorazepate, chlordiazepoxide, diazepam, oxazepam | *MEKC-UV* Fused-silica capillary: 50 cm × 50 μm i.d., BGE: 25 mM borate buffer (pH 9.3) + 50 mM SDS + 15 mM β-CD + 2 M urea, separation voltage: 25 kV, temperature: 25ºC, UV detection at 214 nm | 580-1,510 ng/mL [a] | – | – | [57] |
| Diazepam, clorazepate, chlordiazepoxide, bromazepam, nitrazepam, alprazeolam, flunitrazepam | *Sweeping-MEKC-UV* Fused-silica capillary: 50 cm × 50 μm i.d., BGE: 15 mM borate buffer (pH 9.0) + 20 mM *N*-cetyl-*N*-methyl-pyrrolidinium bromide + 30% v/v methanol, separation voltage: -25 kV, temperature: 25ºC, UV detection at 230 nm | 4.7-9.8 ng/mL [a] | Urine | SPE with Oasis mixed-mode cation exchange cartridge | [58] |
| Alprazolam, clonazepam, diazepam, flunitrazepam, oxazepam, α-hydroxy-alprazolam, 7-AC, 7-AF, nordiazepam, N-demethyl-flunitrazepam, KET, COD, MOR | *Reverse migrating micelles-Sweeping-MEKC-UV* Fused-silica capillary: 50 cm × 50 μm i.d., BGE: 75 mM phosphate buffer (pH 2.5) + 30% v/v methanol, sweeping buffer: 75 mM phosphate buffer (pH 2.5) + 10% v/v methanol + 65 mM SDS, separation voltage: -15 kV, temperature: 25ºC, UV detection at 200 nm | 20-50 ng/mL [b] | Urine | LLE with DCM | [59] |
| COD, MOR, MP KET, alprazolam, clonazepam, diazepam, flunitrazepam, nitrazepam, oxazepam | *LVSS-sweeping-MEKC-UV* Fused-silica capillary: 50 cm × 50 μm i.d., BGE: 50 mM phosphate buffer (pH 2.3) + 10% methanol + 150 mM SDS, separation voltage: -15 kV, temperature: 25ºC, UV detection at 200 nm | 7.5-30 ng/mL [b] | Urine | LLE with EtAc | [60] |

**Table 3.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| Alprazolam, chlordiazepoxide, nitrazepam, triazolam, lorazepam, clonazepam, flunitrazepam, clorazepate, diazepam, prazepam | *CEC-ESI-MS(TOF)* Capillary: 50 cm × 100 µm i.d., 30 cm packed with hexyl acrylate-based porous monolith, mobile phase: 5 mM ammonium acetate (pH 7.0) + 70% v/v acetonitrile, separation voltage: 20 kV, temperature: 25ºC | 0.6-1.8 ng/mL [a] | Urine | LLE with EtAc | [61] |

[a] Standard samples.

[b] Real samples.

second on BZDs, with the CE-based analytical methods currently used being reviewed and summarized in Table 3 [49–60].

*3.1. Barbiturates*

Barbiturates are chemical derivatives of barbituric acid, and are used as sedatives, hypnotics, anticonvulsants, anesthetics and tranquilizers. In recent years, their use decreased markedly, as BZDs replaced barbiturates for the majority of clinical indications. However, the illegal abuse of barbiturates is still widespread, being implicated in cases of murder, suicide and accidents. Commonly abused barbiturates include secobarbital, pentobarbital and amobarbital [48], so analytical methods for their efficient and accurate determination are required.

As shown in Table 3, the separation of barbiturate drugs by CE has been performed by CZE [49–51] and MEEKC [52] using UV as the detection system. In most of these papers, the methods developed have been applied to the analysis of human biological samples. For example, a simple, rapid CZE-based method has been developed for the determination of nine barbiturates in urine, using hexadimethrine bromide as electroosmotic flow modifier [49]. Good intra-day RSDs of the migration times (≤3.84%) and peak areas (≤5.45%), and LODs in the range 0.87–3.5 µg/mL were obtained, without prior sample preparation. Although the authors claimed that the established sensitivity was enough to quantify urine samples at therapeutic levels, the standard cut-offs used in hospitals for barbiturates in urine are

0.3 μg/mL [47]. This means that, in some cases, false negative results could be reported. As a result, this method might have limited use in analytical laboratories and, in this regard, a number of authors have established different strategies to increase the concentration sensitivity of barbiturates.

For example, various preconcentration methodologies have been developed, such as large volume sample stacking (LVSS) and electrokinetic supercharging (EKS) both in CZE [50, 51], and sample stacking induced by reverse migrating pseudostationary phase in MEEKC [52]. Fan *et al.* [50] developed an LVSS-CZE method with polarity switching for the determination of barbiturates in plasma. This technique involves injecting a large volume of sample into the capillary and removing the sample matrix plug out of the capillary by reversing the polarity. Combined with off-line LLE, enrichment factors of up to 203-fold were obtained with UV detection, compared to conventional CZE, resulting in LOQs of 15–57 ng/mL. The method was successfully applied to the analysis of barbiturates in two kinds of forensic specimen (blood and urine), so it can be concluded that this stacking method may be an efficient and useful way for identifying and quantifying these DOAs at very low levels.

EKS in combination with CZE was investigated for the determination of three barbiturates in urine by Botello *et al.* [51]. A 1,050-fold enhancement in detection sensitivity was obtained when the sample was injected at −8.5 kV for 300 s. Sodium chloride (50 mM) was used as leading electrolyte and CHES (100 mM) was used as terminating electrolyte. Blank urine samples spiked with the drugs were analyzed using LLE as a clean-up step prior to analysis, achieving LODs (8–15 ng/mL) with UV detection far below the cut-off values in urine {300 ng/mL [47]}. This method proved suitable for determining trace amounts of these illicit drugs in forensic analysis.

Kadi *et al.* [52] also investigated stacking induced by reverse migrating pseudostationary phase in MEEKC for the determination of phenobarbital and its *p*-hydroxyphenobarbital metabolite in rat urine. When this preconcentration technique is used, the sample stacking and the separation processes take place successively by changing the voltage with an intermediate polarity switching step. Blank rat urine spiked with the drugs was analyzed using SPE as a clean-up step prior to analysis, achieving LODs of 16.8 ng/mL for both analytes under study. The proposed method seems

appropriate for conducting pharmacokinetic studies.

### *3.2. Benzodiazepines*

BZDs are currently among the most frequently prescribed drugs worldwide. They act as anxiolytics, sedatives, hypnotics, amnesics, antiepileptics and muscle relaxants. They are also often used for the treatment of epilepsy, convulsions and many psychiatric disorders. Overdoses of these substances can cause acute toxicity with symptoms, such as drowsiness, dizziness, blurred vision, slurred speech, difficulty breathing, and coma, so it is important to monitor their concentrations in biological samples [48]. However, the physicochemical nature of BZDs may give rise to some problems when using CE as the analytical technique, as these compounds are difficult to ionize, due to their fairly low $pK_a$ values, which makes MEKC the preferred CE separation mode [54–59]. But, these have also been successfully separated by CZE using coated capillaries [53] and CEC [60], as shown in Table 3.

With respect to the detection system, UV has been more widely employed [53–59], although the use of MS has also been reported [60]. With respect to application to biological samples, most of the papers describe the determination of BZDs in urine, using SPE [55, 57] or LLE [58–60] as a sample pretreatment. As can be seen in Table 3, analysis of BZDs has also been reported together with other drugs, such as the opiate-related compounds, hallucinogens and ATSs [58, 59].

However, the determination of BZDs in beverages is also of great interest, since these constitute one of the groups of substances most commonly associated with drug-facilitated crimes, being secretly administered to the victim prior to the assault, usually in alcoholic drinks. In this respect, a rapid CZE method has been developed for the simultaneous determination of nine BZDs in spiked beverages [53]. Because these analytes have very similar hydrophobicity, making them difficult to analyze by CZE, a double coated capillary with poly (diallyldimethylammonium chloride) and then dextran sulfate was employed, which allowed a good baseline resolution between consecutive peaks in a run time of <6.5 min. Peak area repeatability was in the order of 0.9–11% RSD and the migration time repeatability was 0.3–0.8% RSD, making the proposed method useful when a general profile of BZDs in drinks is desired.

However, the gradual removal of dextran sulfate from the capillary wall during analyses led to irreproducible electroosmotic flow, which may be considered a drawback of this strategy. In this work, flushing with dextran sulfate was required approximately every 20 injections in order to overcome this issue.

A somewhat different approach when using MEKC-UV was given by Su *et al.* [54] with the purpose of improving the resolution in the simultaneous analysis of several BZDs. The authors used a chaotropic salt, lithium bis (trifluoromethane-sulfonyl)imide, as the single modifier of the BGE, achieving complete resolution of the BZDs within 16 min without the need to add an organic solvent, in contrast to the need for both surfactant and organic solvent in the conventional MEKC separation. According to the authors, the resolution was improved because the chaotropic anions strongly disrupted hydration of analytes, increasing their hydrophobicity and strengthening their interactions with them. Using 20 mM phosphate with 300 mM lithium bis(trifluoromethanesulfonyl)imide at pH 7.0 as the BGE, the separation was very reproducible with RSDs of the migration times for all the analytes <0.38%, making the proposed method a reliable, simple alternative for improving the separation of this kind of compound.

The same research group also reported enhancement in the separation of BZDs using 1-butyl-3-methylimidazolium-based ionic liquids (ILs) and sodium dodecyl sulfate (SDS) as modifiers in the BGE [55]. In particular, 1-butyl-3-methylimidazoliu bis(trifluoro-methanesulfonyl)imide was the best IL additive for the separation because its anionic moiety interacted favorably with the BZDs. The optimal BGE of 20 mM phosphate at pH 7.0 with 170 mM 1-butyl-3-methylimidazolium bis (trifluoromethanesulfonyl)imide and 10 mM SDS in 55% methanol provided good reproducibility (RSDs of the migration times and peak areas <0.3% and <10%, respectively), and high efficiency (up to 352,000 plates/m), as well as satisfactory peak shapes for all of the analytes. The LODs were 2.74–4.42 μg/mL. Application to biological samples was performed (blank urine spiked with the analytes) after off-line SPE. However, poor sensitivity was achieved and this means that, using the proposed method, single drug intake cannot be detected, which is especially significant if there are legal implications of drug consumption (e.g., in drug-facilitated crimes).

In order to increase the sensitivity of CE for the determination of BZDs, some authors also developed

methodologies in which different preconcentration strategies based on sweeping were used [57–59]. For example, Chiang *et al.* [58] reported a sweeping-MEKC method for the simultaneous determination of different BZDs and ketamine in abusers' urine. For sensitivity enhancement, hydrodynamic sample loading was carried out in this study, combined with stacking with reverse migrating micelles. The capillary was filled with a high conductivity buffer (75 mM phosphate at pH 2.5 in 30% methanol), followed by a large volume of sample in a low concentration buffer (15 mM phosphate at pH 5.0). Simultaneous sweeping and separation was performed in a sweeping BGE (75 mM phosphate with 64 mM SDS at pH 2.5 in 10% methanol). At application of voltage, SDS started to sweep the analytes to the outlet, meanwhile, the analytes decelerated at the boundary between the low conductivity buffer and the high conductivity buffer and formed a narrow zone. The LODs were 20–50 ng/mL.

Alternatively, given that ILs with surfactant properties can form micelles and hence be useful in MEKC, the use of IL-type surfactants as sweeping carriers was proposed for the preconcentration of BZDs during the sweeping-MEKC process [57]. The authors investigated 1-cetyl-3-methylimidazolium bromide and *N*-cetyl-*N*-methylpyrrolidinium bromide as sweeping cationic surfactants and compared them with the most commonly employed cationic surfactant, cetyltrimethylammonium bromide. The experimental results showed that cationic surfactant *N*-cetyl-*N*-methylpyrrolidinium bromide exhibited superior sweeping power compared to 1-cetyl-3-methyl-imidazolium bromide and cetyl-trimethylammonium bromide. Using a BGE consisting of 15 mM borate with 20 mM *N*-cetyl-*N*-methyl-pyrrolidinium bromide at pH 9.0 in 30% methanol, enrichment factors up to 165-fold were obtained with UV detection, compared to conventional MEKC. The LODs achieved were of the order of 4.7–9.8 ng/mL. The applicability of the method for the analysis of urine samples (blank urine spiked with the analytes) was demonstrated, employing SPE as a clean-up step before the analysis. The sweeping-MEKC electropherogram of spiked urine after the SPE is shown in Figure 2.

CEC using a hexyl acrylate-based monolithic column coupled to ESI-(TOF)MS, which offers the advantage of accurate mass measurements, has also been demonstrated to be effective for separation and determination of BZDs in urine [60].

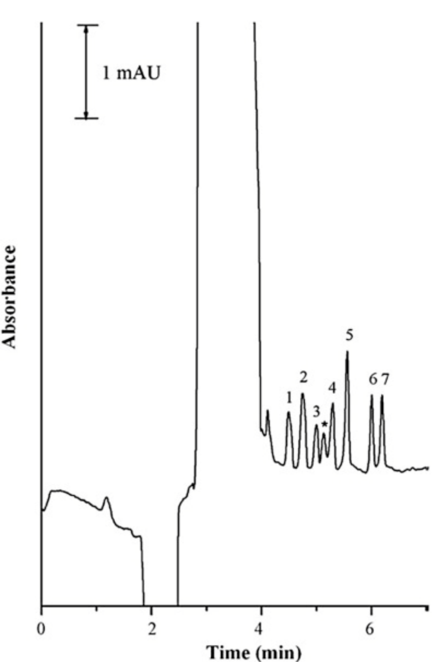


**Figure 2.** Electropherogram of urine spiked with the analytes at a concentration of 1 μg/mL analyzed by sweeping-MEKC-UV after SPE. Peak identification: (1) bromazepam, (2) alprazeolam, (3) flunitrazepam, (*) unknown peak, (4) chlordiazepoxide, (5) diazepam, (6) clorazepate and (7) nitrazepam. Reproduced from [57] with the permission of Elsevier, ©2010.

Using a mobile phase of 5 mM ammonium acetate at pH 7.0 in 70% acetonitrile, RSD values were obtained of 1.4–2.3% for retention times and 1.1% and 9.2% for relative areas. Stacking of the analytes at the head of the stationary phase was performed, consisting of the injection of a large volume of sample present in aqueous solution, enabling an improvement in sensitivity of 75–140-fold. This strategy enabled the quantification of these drugs down to 1 ng/mL after off-line LLE, which is far below the cut-off levels in urine {300 ng/mL [47]}. Although these were probably preliminary results, as complete method validation was not carried out, this work shows the potential of coupling CEC with ESI-(TOF)MS for determining very low levels of benzodiazepines in urine, which could allow quantification of single drug intake useful in cases involving drug-facilitated crimes.

## 4. Opium-related compounds

Derived from the poppy plant, opiates are powerful drugs that have been used for centuries to relieve pain. Also known as narcotics, opiates can be natural or synthetic. Natural opiates include morphine, codeine and thebaine. Other substances, called opioids, are manmade and are also used to treat chronic or severe pain. These substances include pethidine, oxycodone, hydrocodone, fentanyl, buprenorphine, and methadone. Heroin is a manufactured opioid and has no medicinal uses. It is mainly used for its ability to give the user a feeling of euphoria [16]. Opium-related substances are highly addictive and overuse can cause overdose and potentially death. Since their abuse

has risen substantially worldwide, methodologies able to analyze opioids need to be developed. Table 4 reviews and summarizes the CE-based analytical methods currently used for this purpose [62–75], two of which involve the determination of the opium-related compounds together with ATSs [63, 71].

As shown in Table 4, toxicological analysis of opiate-related compounds has been performed using CZE [61–64, 73, 74], CEC [65–67], MEKC [68, 70–72] and non-aqueous CE [69] with detection by UV [62, 65, 67, 68, 70–72, 74] and MS [63, 64, 66, 69, 73]. For example, Zhang and co-workers [64] reported an approach for the analysis of heroin and its related alkaloids using charged polymer protected gold nanoparticle (AuNP)-coated capillaries with CZE-ESI-(IT)MS. The use of AuNP-coated capillaries exhibited good efficiency in separation (up to 498,052 plates/m), peak shape and analysis time with satisfactory run-to-run and capillary-to-capillary repeatabilities, obtaining RSDs of migration time in the range 0.43–0.62% and RSDs of peak area in the range 1.49–4.68%. The capillary coating procedure proved to be simple and fast (30 min) without requiring heating. This method seems to be reasonably suitable for the identification of the geographical origin of illicit samples.

The application of a pressure via a micro-HPLC pump (a pressure of 1,000 psi and a flow rate of 0.05 mL/min) also allowed high resolution combined with a considerable reduction of the analysis time in the determination of five opium alkaloids in *Pericarpium papaveris* samples by CEC using a poly(glycidyl metha-crylate-*co*-ethylene dimeth-acrylate-*co*-3-sulfopropyl methacrylate potassium salt) monolithic column prepared in house [65]. With UV detection, the LODs were in the range 1.5–6.0 μg/mL, which are sufficient for detection of these compounds in cases involving drug possession and seized drugs. Good intra-day RSDs (≤1.94%), inter-day RSDs (≤3.49%) and column-to-column RSDs (≤5.24%) for the retention time were obtained. However, the preparation procedure of the monolithic column is quite time consuming, which may be considered a drawback of this method

The modification of the inner surface of the capillary in open-tubular CEC with carboxymethyl chitosan showed that it counteracts adsorption of the analytes and, most importantly, stabilizes the electro-osmotic flow when four opium alkaloids (morphine, thebaine, papaverine and narcotine) were baseline separated in phosphate

**Table 4.** Overview list of capillary electrophoresis (CE) methodologies for determination of opium-related compounds.

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| Pethidine, MTD | *CZE-ECL* Fused-silica capillary: 67.5 cm × 25 μm i.d., BGE: 30 mM phosphate buffer (pH 6.0), separation voltage: 14 kV, temperature: 25ºC | 0.5 μM [a] | – | – | [62] |
| MOR, COD, oripavine, thebaine | *CZE-UV* Fused-silica capillary: 50 cm × 50 μm i.d., BGE: 100 mM Tris (pH 2.8) + 30 mM hydroxypropyl-β-CD, separation voltage: 25 kV, temperature: 25ºC, UV detection at 214 nm | 2.5 10-6 M [a] | Process liquor | – | [63] |
| AP, EP, MTD, pethidine, COD, tetracaine, heroin | *CZE-ESI-MS(Q)* Fused-silica capillary: 70 cm × 50 μm i.d., BGE: 20 mM ammonium acetate (pH 9.0), separation voltage: 22 kV, temperature: 20ºC | 0.40-1.0 ng/mL [a] | Urine | Protein precipitation | [64] |
| MOR, 6-AC, 6-AM, COD, thebaine, heroin, papaverine, narcotine | *CZE-ESI-MS(IT)* Charged polymer-protected gold nanoparticles-coated capillary: 68 cm × 50 μm i.d., BGE: 120 mM ammonium acetate (pH 5.2) + 13% v/v methanol, separation voltage: -20 kV, temperature: 20ºC | | | | [65] |
| Narcotine, papaverine, thebaine, COD, MOR | *Pressure-assisted CEC-UV* Capillary: 55 cm × 100 μm i.d., 30 cm packed with polymeric monolith, mobile phase: 5 mM phosphate buffer (pH 4.0) + 90% v/v acetonitrile, separation voltage: -13 kV, temperature: 25ºC, UV detection at 224 | 1,500-6,000 ng/mL [b] | Pericar-pium papa- | Ultrasound extraction with MeOH for 1 h and then soaked for 24 h | [66] |

**Table 4.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| MTD, pethidine, fentanyl, MOR, diamorphine | *Pressure-assisted CEC-ESI-MS(Q)* Silica-based monolithic capillary: 56 cm × 100 μm i.d., mobile phase: 20 mM ammonium acetate (pH 6.0) + 65.5% v/v acetonitrile, separation voltage: 25 kV, temperature: 25ºC | 2.0-80 nmol/L [a] | Urine | Centrifugation with MeOH at 4,500 rpm for 20 min | [67] |
| Morphine, thebaine, narcotine, papaverine | *Open-tubular CEC-UV* Capillary: 60 cm × 50 μm i.d., covalently modified with hydrophilic polysaccharide, mobile phase: 50 mM phosphate buffer (pH 6.0), separation voltage: -15 kV, temperature: 25ºC, UV detection at 214 nm | – | – | – | [68] |
| Paclitaxel, MOR, COD | *MEKC-UV* Fused-silica capillary: 57 cm × 75 μm i.d., BGE: 20 mM borate buffer (pH 9.2) + 60 mM SDS + 5% v/v methanol, separation voltage: -25 kV, temperature: 20ºC, UV detection at 212 nm | 30-90 ng/mL [a] | Urine | Centrifugation at 5,000 rpm for 5 min | [69] |
| Fentanyl derivatives | *Non-aqueous CE-ESI-MS²(IT)* Fused-silica capillary: 80 cm × 50 μm i.d., BGE: non-aqueous solution of ammonium acetate 200 mM + 90% v/v acetonitrile, separation voltage: 28 kV, temperature: 20ºC | 0.5 ng/mL [a] | Seized samples | Dilution | [70] |

**Table 4.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| MOR, COD, normorphine, M3G, M6G | *CSEI-Sweeping-MEKC-UV* Fused-silica capillary: 40 cm × 50 μm i.d., BGE: 25 mM phosphate buffer (pH 2.5) + 22% v/v methanol + 100 mM SDS, separation voltage: -20 kV, temperature: 25ºC, UV detection at 200 nm | 10-35 ng/mL [a] | Urine | SPE with BondElut Certify cartridges | [71] |
| MTD, EDDP, AP, MP, MOR | *CSEI-Sweeping-MEKC-UV* Fused-silica capillary: 30 cm × 50 μm i.d., BGE: 100 mM phosphoric acid (pH 4.0) + 100 mM SDS + 20% v/v tetrahydrofuran, separation voltage: -15 kV, temperature: 25ºC, UV detection at 214 nm | 0.2-0.4 ng/mL [a] | Serum | LLE with EtAc | [72] |
| Heroin, MOR, COD, 6-AM | *CSEI-sweeping-MEKC-UV* Fused-silica capillary: 38 cm × 50 μm i.d., BGE: 20 mM phosphate buffer (pH 2.5) + 80 mM SDS, separation voltage: 20 kV, temperature: 25ºC, UV detection at 214 nm | 10 ng/mL [b] | Urine | LLE with DCM: *n*-propanol, 85:15 v/v | [73] |
| EDDP, 6-AM, COD, DHCOD | *In-line SPE-CZE-ESI-MS(IT)* Fused-silica capillary: 100 cm × 50 μm i.d., BGE: 60 mM ammonium acetate (pH 3.8), separation voltage: 30 kV, temperature: 25ºC | 13-210 ng/mL [b] | Urine | Dilution | [74] |
| MOR, COD, 6-AM | *pH-mediated stacking-CZE-UV* Fused-silica capillary: 50 cm × 75 μm i.d., BGE: 100 mM phosphate buffer (pH 3.0) + 20% v/v methanol+ 5% v/v n-propanol, separation voltage: 25 kV, temperature: 25ºC, UV detection at 214 nm | 7 ng/mL [a] | Saliva | Dilution or LLE | [75] |

[a] Standard samples.

[b] Real samples.

buffer (50 mM, pH 6.0) with column efficiencies up to 132,000 plates/m [67]. Although good repeatability was gained with RSDs of the migration time less than 1.3% for run-to-run and less than 3.2% for day-to-day, and RSDs of peak area less than 5.6% for run-to-run and less than 8.8% for day-to-day, no application to real samples was proposed.

In recent years, many illegal drug laboratories started to produce synthetic derivatives of fentanyl, which are more potent that morphine, and, as a result, abuse of these drugs is rapidly growing. With this in mind, a non-aqueous CE-$MS^2$ method was proposed for the trace identification of these illicit drugs, which were detected down to the nanomolar level (0.5 ng/mL for fentanyl) [69]. The method was successfully applied for the analysis of three samples from forensic casework. From an electrophoretic point of view, the system is quite straightforward and, although it is not particularly innovative, its performance seems to be suitable for the stated purpose.

In order to increase the sensitivity in CE and, consequently, decrease LODs for the determination of opiates/opioids, some authors have developed methodologies using different preconcentration techniques, such as sweeping [70–72], in-line SPE [73] and pH-mediated staking [74]. For example, Meng *et al.* [74] used both acid-mediated and base-mediated stacking in CZE-UV for the analysis of codeine, morphine and 6-acetylmorphine in saliva. 100 mM hydrochloric acid and 12.5% ammonium hydroxide in 50% methanol were used to create the respective pH pulses in acid- and base-mediated stacking, respectively. The methods resulted in a 1,000-fold sensitivity increase compared with normal hydrodynamic injection, with LODs ≤7 ng/mL. In acid-mediated stacking, the sample preparation involved dilution with methanol while, in basic-mediated stacking, an LLE step was necessary to make the analytes go into the acid matrix. Unfortunately, acid-mediated stacking displayed a considerable background noise and, in the case of basic-mediated stacking, although cleaner electropherograms were obtained, the separation efficiency was lower in comparison to acid-mediated stacking. Despite this, preconcentration factors were higher than those in the analysis of the same substances of abuse in urine samples by CSEI-sweeping MEKC [72].

Methadone is an opioid that is primarily used therapeutically in maintenance treatment of heroin addicts, although, in recent years there

has been abuse of this compound on the drug scene. With this in mind, Wang *et al.* [71] developed a sweeping-MEKC method for the determination of methadone and its metabolites in serum using off-line LLE and injection under CSEI conditions for the sample clean-up and preconcentration of the analytes, providing LODs in the range 0.2–0.4 ng/mL with UV detection. Analytical precision and accuracy were fairly acceptable, with RSDs and relative errors <19% in real samples in both intra-day and day-to-day experiments. The method was successfully applied for monitoring the studied drugs in heroin addicts, proving to be a valuable, reliable tool for toxicological analysis, therapeutic drug monitoring, drug rehabilitation programs and doping control.

Since methadone and its metabolites are chiral compounds, enantiomeric separation of these drugs plays a fundamental role in the determination of synthetic pathways and for impurity profiling of the drug. In this respect, a review was published describing the most recent papers on the enantioseparation of methadone and its metabolites [45]. In short, native CDs and CD derivatives (sub-section 2.1) are the most popular chiral selectors for the enantiomeric analysis of methadone and its metabolites in biological samples. The number of newly synthesized CD derivatives is increasing and their application is continually being investigated. However, none of these investigations has yet clearly proved to offer significant advantages over the others.

## 5. Cannabinoids

Cannabinoids are compounds derived from *Cannabis sativa* (marijuana). The primary psychoactive component of marijuana is $\Delta^9$-tetrahydrocannabinol that is found in the flowering tops, leaves and resin of the plant. Once ingested, it is primarily metabolized in the body to 11-hydroxy-$\Delta^9$-tetrahydrocannabinol, which is further oxidized to 11-nor-9-carboxy-$\Delta^9$-tetrahydrocannabinol. Other cannabinoids are also present in significant amounts, including cannabidiol and cannabinol. Marijuana consumption can cause euphoria, hallucination, difficulties in concentration and impairment of memory, among other symptoms. Because of its increasingly widespread abuse worldwide, there is a need for rapid, sensitive and accurate analytical methods for the determination of cannabinoids.

To the best of our knowledge, in the period covered by this review, only two papers were reported on the

**Table 5.** Overview list of capillary electrophoresis (CE) methodologies for determination of determination.

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample treatment* | *Ref.* |
|---|---|---|---|---|---|
| Δ⁹-tetrahydrocannabinol, 11-hydroxy-Δ⁹-tetrahydrocannabinol, 11-nor-9-carboxy-Δ⁹-tetrahydrocannabinol | *Sweeping-MEKC-UV* Fused-silica capillary: 60 cm × 50 μm i.d., BGE: 25 mM phosphate buffer (pH 2.6) + 40% v/v methanol + 75 mM SDS, separation voltage: -20 kV, temperature: 25 ºC, UV detection at 210 nm | 17.2-23.3 ng/mL | Urine | SPE with Bond Elut Certify cartridge | [76] |
| Δ⁹-tetrahydrocannabinol, 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid, 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid glucuronide | *CZE-ESI-MS(QqQ)* Fused-silica capillary: 85 cm × 50 μm i.d., BGE: 40 mM ammonium formate (pH 6.4), separation voltage: 30 kV, temperature: 25 ºC | 50 ng/mL | Urine | Dilution | [77] |

application of CE for the determination of these DOAs {Table 5 [75, 76]}. Su *et al.* [75] described a sensitive method that utilizes both off-line and on-line procedures in MEKC for the simultaneous determination of Δ⁹-tetrahydrocannabinol and its metabolites in urine. In particular, the authors used SPE for off-line preconcentration and clean-up of the sample and on-line preconcentration based on sweeping, resulting in enhancement factors of up to 200-fold compared to conventional MEKC with UV detection. The LODs for urine samples were in the range 17.2–23.3 ng/mL, which are below the cut-off levels in urine cut-off levels in urine {50 ng/mL [47]}. However, the overall analysis time including sample preparation (off-line SPE) was ~80 min, which makes the method less appealing for routine analysis.

Recently, a faster (approx. 30 min), simpler method was developed for the direct analysis of Δ⁹-tetrahydrocannabinol's metabolites in urine by CZE-ESI-(QqQ)MS [76]. Due to the use of a highly selective detector, such as (QqQ)MS, the only pretreatment needed for urine sample was dilution with methanol and centrifugation achieving LODs of 50

ng/mL, which proved once more the potential of the coupling between CE and (QqQ)MS for the analysis of low levels of DOAs. Analytical precision was reasonably acceptable with RSDs ≤0.36% for migration times and ≤25.0% for areas in real samples, in both intra-day and day-to-day experiments. The proposed method was successfully applied to the analysis of urine samples collected from cannabis users.

## 6. New designer drugs

Recent abuse of new designer drugs (synthetic cathinones, synthetic cannabinoids, piperazines, benzofurans, thiophenes and structural analogues of methylphenidate) gave rise to the need for sensitive, reliable, reproducible analytical methods for their determination in different kinds of matrix [77]. In 2010, Wohlfarth *et al.* [78] reviewed analytical protocols proposed in the past 10 years for the analysis of these compounds in biological matrices, including CE-based methods. With this in mind, this section will focus on papers {Table 6 [79–86]} related to the determination of new designer drugs by CE published after 2010 up to the present in different types of samples.

CZE-UV with CDs as chiral selectors for enantioseparations [79–81] and MEKC-UV with SDS as surfactant to enhance the separation [83] have been successfully applied for the analysis of new designer drugs in confiscated pills [79] and herbal blends [83]. However, in conjunction with ESI–MS, CDs and SDS may cause significant signal suppression. In order to overcome this issue, the application of a novel interface was proposed, referred to as sheathless CE, allowing the coupling of CE with ESI-MS in a single dynamic process within the same device for the MEKC-MS analysis of various cathinones [82]. A detailed description of this interface has been given by Haselberg *et al.* [87]. The approach proved to be suitable for reducing the ion suppression due to the presence of highly sulfated-γ-CD and 18-crown-6-tetracarboxylic acid used in this work as the BGE with good chiral resolution for the studied compounds within reasonable analysis times (20 min). However, further analysis and validation are required before being adopted by analytical laboratories to carry out enantioselective determinations on a routine basis.

As an alternative to the use of these conventional surfactants, salts of perfluorocarboylic acid have been used as the BGE in MEKC-ESI-MS for the determination of several cathinones in urine [84]. According to that work, the BGE used to form

**Table 6.** Overview list of capillary electrophoresis (CE) methodologies for determination of new designer drugs.

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| 1-(2-Chloro-phenyl)piperazine, 1-(3-chloro-phenyl)piperazine, 1-(4-chloro-phenyl)piperazine | *CZE-UV* Fused-silica capillary: 60 cm × 50 µm i.d., BGE: 20 mM phosphoric acid (pH 2.5) + 10 mM α-CD, separation voltage: 25 kV, temperature: 25 ºC, UV detection at 236 nm | 2,000-3,000 ng/mL [a] | Confiscated pills | Ultrasound extraction with MeOH for 15 min | [80] |
| 7 Benzofurys, 4 synthetic cathinones, 2 diphenidines, ethylphenidate, methiopropamine, thiothinone | *CZE-UV* Fused-silica capillary: 60 cm × 50 µm i.d., BGE: 50 mM ammonium acetate (pH 4.5) + 15 mM sulfobutylether-β-CD + 10% v/v acetonitrile, separation voltage: 25 kV, temperature: 25 ºC, UV detection at 230 and 280 nm | – | – | – | [81] |
| 12 Synthetic cathinones derivatives | *CZE-UV* Fused-silica capillary: 57.5 cm × 50 µm i.d., BGE: 100 mM phosphate buffer (pH 2.5) + 10 mM β-CD, separation voltage: 25 kV, temperature: 25 ºC, UV detection at 206 nm | 4.2-7.0 ng/mL [a] | – | – | [82] |
| | *CZE-ESI-MS(TOF)* Fused-silica capillary: 57.5 cm × 50 µm i.d., BGE: 50 mM phosphate buffer (pH 2.5) + 0.6% v/v highly sulfated-β-CD, separation voltage: 25 kV, temperature: 25 ºC | 1.0-11.0 ng/mL [a] | – | – | |

**Table 6.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| 6 Synthetic cathinones derivatives, AP, MP | *CZE-Sheathless CE-MS(IT)* Fused-silica capillary: 90 cm × 20 µm i.d., BGE: 15 mM 18-crown-6-tetracarboxylic acid + 0.125% v/v highly sulfated-γ-CD, separation voltage: 25 kV, temperature: 25 ºC | – | – | – | [83] |
| 9 Synthetic cannabinoids | *MEKC-UV* Fused-silica capillary: 40 cm × 30 µm i.d., BGE: 25 mM borate buffer (pH 8.0) + 30 mM SDS + 20% v/v *n*-propanol, separation voltage: 30 kV, temperature: 25 ºC, UV detection at 220 nm | 1,000-1,500 ng/mL [a] | Herbal blends | Ultrasound extraction with MeOH for 15 min and centrifugation at 3,500 rpm for 10 min | [84] |
| 12 Synthetic cathinones derivatives | *MEKC-ESI-MS/MS(QqQ)* Fused-silica capillary: 90 cm × 50 µm i.d., BGE: 100 mM perfluooctanoic acid + 200 mM ammonium hydroxide, separation voltage: 22 kV, temperature: 25 ºC | 10-78 ng/mL [b] | Urine | SPE with Supel-Select strong cation exchange cartridge | [85] |
| 12 Synthetic cannabinoids | *MEKC-ESI-MS/MS(QqQ)* Fused-silica capillary: 90 cm × 50 µm i.d., BGE: 50 mM perfluooctanoic acid (pH 9.0) + 20% v/v acetonitrile/water, separation voltage: 30 kV, temperature: 25 ºC | 6,500-30,200 ng/g [a] | – | Ultrasound extraction with MeOH for 10 min | [86] |
| 10 Synthetic cathinones derivatives | *FASI-CEC-UV* Capillary: 100 µm i.d., packed with amylose *tris*(5-chloro-2-methylphenylcarbamate), mobile phase: 250 mM sodium acetate (pH 9.0) + 89% v/v acetonitrile + 10% v/v methanol, separation voltage: 10 kV, temperature: 20 ºC, UV detection at 206 nm | 25-100 ng/mL [a] | – | – | [87] |

[a] Standard samples.

[b] Real samples.

micelles did not affect the electron-ionization efficiency of MS, in contrast to the use of SDS and CDs. Blank urine spiked with the drugs was analyzed using SPE as clean-up prior to analysis, reaching LODs of 10–78 ng/mL. Figure 3 shows the reconstructed selected reaction monitoring electropherograms of the blank and spiked urine after the SPE. A similar method for the analysis of various synthetic cannabinoids in herbal blends was also presented with satisfactory results [85]. Nevertheless, the widespread application of these approaches might be limited because the salts of perfluorocarboylic acid are toxic surfactants, but, in any case, the developed methods represent a good tool for a rapid screening of new psychoactive drugs in illegal drug laboratories as well as in drug seizures.

CEC with UV [86] was also proposed for the analysis of cathinones using a fused-silica capillary column packed with amylose *tris* (5-chloro-2-methylphenylcarbamate), also called Sepapak 3 or Lux Amylose-2. To obtain enhanced sensitivity, FASI for on-line sample preconcentration was performed, achieving LODs of 25–100 ng/mL. Good intra-day RSDs (≤1.6%), inter-day RSDs (≤2.3%) and column-to-column RSDs (≤2.9%) for the retention time were obtained. However, no complete method validation or application on real samples was carried out.

## 7. Concluding remarks

This review compiles and summarizes the most recent developments in determining DOAs and their metabolites using CE. Although CE still has some important drawbacks, mostly due to its low sensitivity, CE-based techniques have often been successfully used for the analysis of these substances in a variety of matrices, shown by the large number of applications developed in recent years.

The lack of sensitivity has been overcome in several ways, using chromatographic and electrophoretic preconcentration techniques or a combination of these two procedures. However, these steps also involve certain disadvantages, such as more complicated and time consuming procedures, lower reproducibility or, in some cases, environmental damage Therefore, in this respect, future trends should focus on the study and the application of more sophisticated and more efficient on-line preconcentration strategies in order to achieve better analytical performance.

With respect to the samples tested

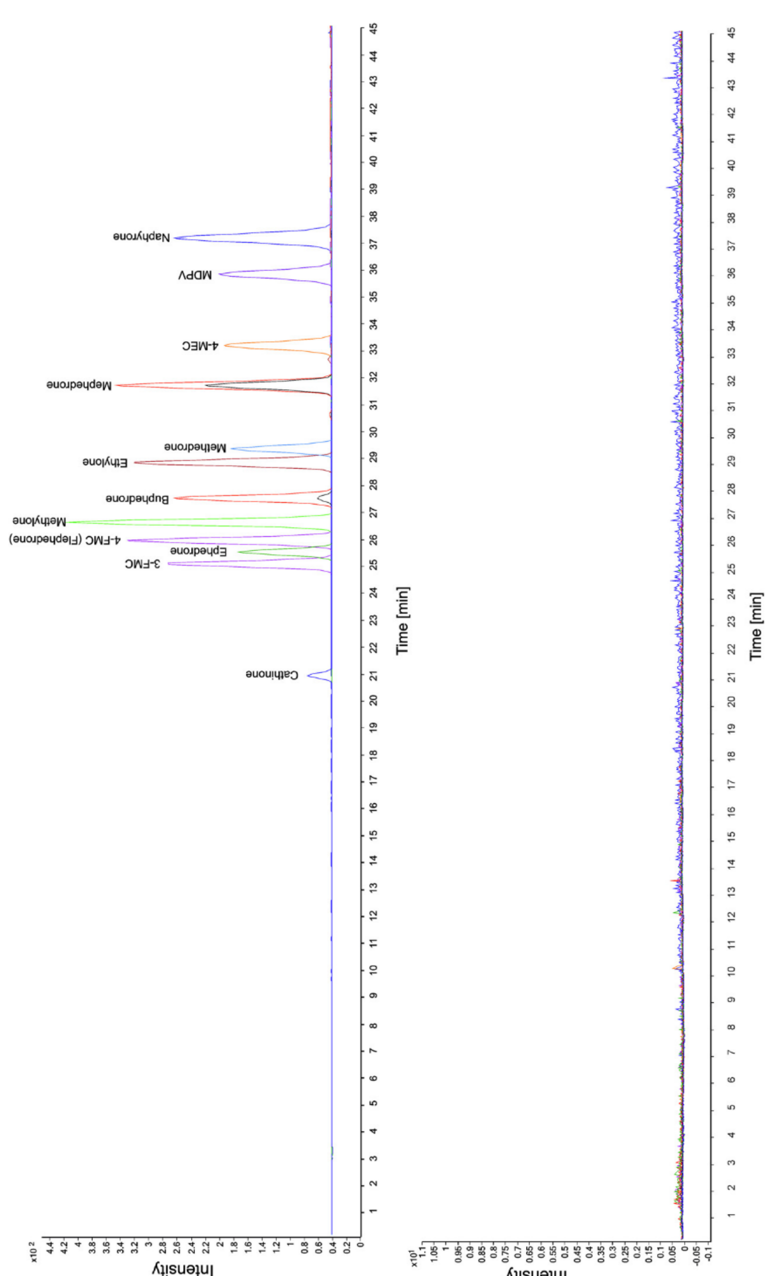


**Figure 3.** Selected reaction monitoring electropherograms recorded from spiked urine with the analytes at concentration of 300 ng/mL and blank urine after SPE. Reproduced from [84] with the permission of Elsevier, ©2010.

to determine substances of abuse, existing literature mainly focuses on biological samples, with urine being the most commonly used matrix. In this sense, further work is needed in developing CE-based tools suitable for analyzing other biological fluids, as well as other different types of matrix, such as environmental.

As is well known, MS offers an alternative to the sensitivity enhancement in CE, so the importance of coupling this detection system with CE will continue to grow in toxicological analysis, so further improvements are needed in terms of combining on-line preconcentration strategies and CE separation with MS detection, as are considerable developments in terms of instrumentation and methodology of CE-MS interfacing.

Lastly, very few of the reported papers include complete method validation in accordance with commonly accepted international guidelines [88], which is a major drawback, since it impacts negatively on the reliability of the methods themselves. With this in mind, the future challenge is the development of CE-based procedures that allow method application with sensitivity, precision and reliability compared to those commonly obtained by chromatographic techniques.

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### 1.3.2. Enantioselective determination of chiral drugs of abuse by capillary electrophoresis

Nowadays, it is well documented that many DOAs are chiral and occur in two enantiomers {*R* and *S*, according to IUPAC [124]} that can display significantly different pharmacological and toxicological effects, resulting in one isomer being accepted for therapeutic use, while the other may be an illicit or banned substance [125, 126]. An example of this is methorphan, a synthetic analogue of codeine. *S*-methorphan is an antitussive drug commonly utilized in numerous over-the-counter cough and cold medicines, whereas *R*-methorphan is a strong narcotic analgesic that has a high potential for abuse and is subject to international control [127]. Another example is methamphetamine, a widely abused psychostimulant. *S*-methamphetamine is much more potent than its partner and causes a majority of the effects associated with the drug. It is a controlled substance and used for attention deficit hyperactivity disorder treatment on prescription. *R*-methamphetamine, on the other hand, is the active ingredient in the over-the-counter nasal decongestant Vicks Vapor Inhaler. In addition, *R*-methamphetamine is also a metabolite of certain therapeutic drugs, such as selegiline that is used for the treatment of Parkinson's disease and dementia [128]. Within this context, chiral analysis of DOAs and their metabolites in biological samples is essential to distinguish between licit and illicit consumption accurately, which is especially useful when legal implications are involved.

Moreover, enantiomeric determination of DOAs in seized samples is also of great interest because valuable data can be obtained about the synthesis pathway used, the precursor substance and the origin of a sample [125, 126, 129], which is particularly relevant for profiling clandestine drug labs. For example, the presence of racemic amphetamine (an equal mixture of *R*- and *S*-enantiomers) suggests synthesis from an achiral starting material such as phenylacetone, whereas a single enantiomer suggests stereoselective reduction of norephedrine or norpseudoephedrine [130]. Drugs such as methamphetamine, amphetamine, 3,4-methylenedioxymethamphetamine (ecstasy), methcathinone, phencyclidine, lysergic acid diethylamide and fentanyl are often manufactured illicitly in clandestine labs. Therefore, chiral analysis is a useful tool for linking drug batches, production sites, precursor chemical supply chains and distribution networks, among others. Another application of chiral separations is the evaluation of the metabolism of DOAs in humans. Knowledge on this issue will be highly beneficial for the correct interpretation of drug testing results.

Of the different analytical techniques developed so far to separate chiral compounds (e.g., LC, GC, etc.), CE represents an inexpensive and versatile technique for the resolution of enantiomers because, as is widely known, enantioselective CE

separations are achieved by simply dissolving a chiral selector in the running buffer [124]. This procedure enables easy and fast preparation compared to the modifications of the stationary phase necessary in LC and GC chiral separations [131]. Several chiral selectors are currently available and can be used in CE enantioseparations, such as cyclodextrins (CDs), crown ethers, cyclofructans, proteins, polymeric surfactants and macrocyclic antibiotics, among others [124, 132].

Within this scenario, CE has also found numerous applications in the analysis of different chiral DOAs (e.g., amphetamines, synthetic cathinones, methadone, ketamine, barbiturates, etc.) in biological samples, such as urine, plasma, blood, saliva and hair, and other type of samples, such as street-grade tablets, banknotes, kraft paper, plastic bags, silver paper and drug seizures, which have been thoroughly discussed by various authors [133-140]. These reviews cover the most relevant chiral CE-based approaches proposed between 1995 and 2015, providing information on the chiral selector, CE buffer, detection conditions and capillary used in the analysis of different chiral compounds, including DOAs. Two of these, however, mainly focus on the application of different CDs as chiral selectors in CE for the same purpose [138, 139]. In view of this, this section gives a brief overview of the improvements and innovations performed in CE for the enantioselective analysis of DOAs, including the research works that have appeared in the literature since 2015 {Table 12 [118, 121, 127, 131, 141, 142]}.

**Table 12.** Recent applications of chiral CE in the analysis of DOAs (since 2015).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| DHNK, HNK, KET, NKET | *CZE-UV* Fused-silica capillary: 45 cm × 50 μm i.d.; BGE: 100 mM phosphate (pH 3.0) + 0.66% (w/v) HS-γ-CD; separation voltage: 20 kV; temperature: 20ºC; UV detector at 200 nm | LOQ: 10 ng/mL [b] | Animal plasma and serum | 50 μL; LLE with DCM and centrifugation; Evaporation/ reconstitution | [141] |
| AP, cathinone, nor-mephedrone, pregabalin | *CZE-ESI-MS* Fused-silica capillary: 25 cm × 5 μm i.d.; BGE: 10 mM 18-C-6-TA; separation voltage: -25 kV; temperature: 25°C | – | – | – | [131] |

**Table 12.** (Continued).

| *Substance* | *CE conditions and detection* | *LOD* | *Sample* | *Sample pretreatment* | *Ref.* |
|---|---|---|---|---|---|
| KET, NKET | *FASI-CZE-UV* Fused-silica capillary: 45 cm × 50 μm i.d.; BGE: 15 mM Tris phosphate (pH 2.5) + 0.1% (w/v) HS-γ-CD; separation voltage: 20 kV; temperature: 20°C; UV detector at 200 nm | 0.08 ng/mg [b] | Hair | 50 mg; Washing with SDS, water, MeOH; Digestion with 100 mM HCl (overnight at 37°C) and LLE with hexane/EtAc (50:50, v/v); Evaporation/reconstitution | [118] |
| Methorphan and morphonal enantiomers | *FASI-CZE-UV* Fused-silica capillary: 60 cm × 50 μm i.d.; BGE: 150 mM phosphate (pH 4.4) + 5 mM HP-β-CD + 30% (v/v) MeOH; separation voltage: 25 kV; temperature: 15°C; UV detector at 200 nm | 8 ng/mL [b] | Blood | 500 μL; LLE with hexane/ EtAc (1:1, v/v); Injection into the system | [127] |
| MTD | *FASI-CZE-UV* Fused-silica capillary: 50 cm × 50 μm i.d.; BGE: 150 mM phosphoric acid-TEA (pH 2.5) + 30% (v/v) MeOH + 0.8% (w/v) CM-β-CD; separation voltage: 25 kV; temperature: 15°C; UV detector at 200 nm | LLOQ: 150 ng/mL [b] | Exhaled breath condensate | Direct injection into the system | [142] |
| MP, AP | *CSEI-sweeping-MEKC-UV* Fused-silica capillary: 64.5 cm × 50 μm i.d.; BGE: 100 mM phosphate (pH 2.7) + 20% (v/v) MeOH + 20% (w/v) HS-γ-CD + 20 mM SDS; separation voltage: -18 kV; temperature: 25°C; UV detection at 195 nm | 0.07-0.09 ng/mL [a] | Hair | 3 mg; Incubation in 1,000 mM NaOH (30 min, 70°C) and LLE with cyclohexane; Evaporation/reconstitution | [121] |

See Appendix I for the key to abbreviations.

[a] Standard samples.

[b] Real samples.

Of the different chiral selectors mentioned above, CDs and their derivatives are the most frequently used for the enantiomeric resolution of DOAs by CE due to their good solubility, availability, low toxicity and low UV absorbance. Figure 14 shows a general view of the number of applications of the CDs used collected in the review papers [138] and [139], and in Table 12 [118, 121, 127, 131, 141, 142].

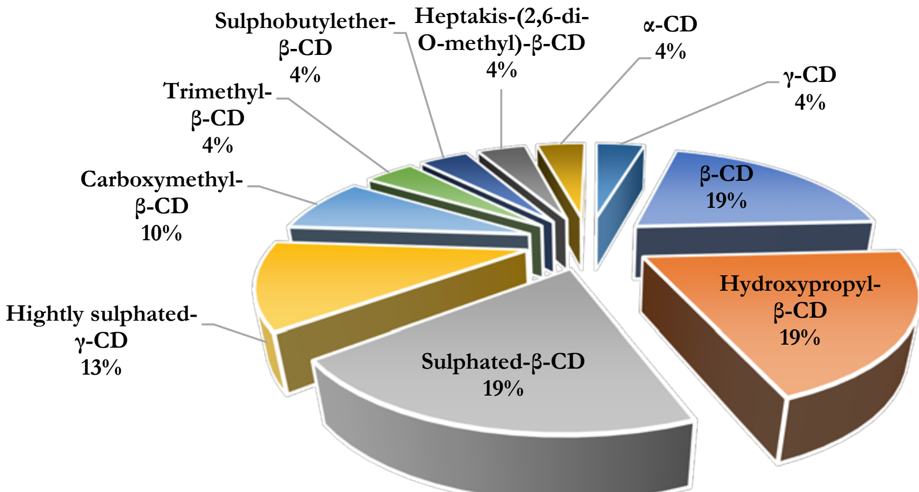



**Figure 14.** General view of the most commonly used CDs in chiral CE analysis of DOAs {data obtained from the number of applications reported in [138], [139] and Table 12 [118, 121, 127, 131, 141, 142]}.

As can be seen from the figure, β-cyclodextrin (CD) and hydroxypropyl-β-CD, which are neutral, and carboxymethyl-β-CD, highly sulphated-γ-CD and sulphated-β-CD, which are anionic, have been the most employed CDs for the chiral separation of DOAs by CE. Because there is no consensus on which CD performs best, different CDs are generally examined to find out the most suitable for the intended purpose. For example, Merola *et al.* [143] investigated the ability of β-CD, highly sulphated-γ-CD and a mixture of both in 100 mM phosphate at pH 2.5 for the enantioseparation of synthetic cathinones. The separations obtained using highly sulphated-γ-CD were not as efficient as those obtained with β-CD. Meanwhile, Mohr *et al.* [144] tested β-CD, carboxymethyl-β-CD, hydroxypropyl-β-CD, sulphated-β-CD and γ-CD in 50 mM ammonium acetate at pH 4.5 for the chiral separation of the same substances, obtaining the best results in terms of resolution with the negatively charged sulphated-β-CD.

Another observation from the figure is that the native β-CD and its derivatives (hydroxypropyl-β-CD, carboxymethyl-β-CD, sulphated-β-CD, trimethyl-β-CD, sulphobutylether-β-CD, and others) have been the most commonly used CDs, probably because the size of their cavity matches the size of many DOAs better. In the case of the negatively charged derivatives (carboxymethyl-β-CD, sulphated-β-CD, sulphobutylether-β-CD), the ionic interactions might also contribute to the desired separation [139].

Chiral crown ether derivatized with carboxylic groups, namely 18-crown-6-tetracarboxylic acid, is another compound used in CE for the resolution of enantiomers of DOAs, although to a lesser extent [131, 145]. It has been shown to be suitable, e.g., in the enantioseparation of amphetamine, cathinone, nor-mephedrone and pregabalin, providing resolution values in the range of 1.3-3.8 [131].

When a single CD is not able to separate the enantiomers, a second CD derivative can be added to the BGE and, in some cases, the required separation might be achieved. Such mixtures are referred to as dual systems. In this regard, dual systems consisting of two neutral CDs [β-CD/dimethyl-β-CD, β-CD/heptakis-(2,6-di-*O*-methyl)-β-CD)] and, in a second case, consisting of two negatively CDs (sulphobutylether-β-CD/sulphated-β-CD), were studied in the separation of several chiral amphetamines, demonstrating to be efficient for the enhancement of enantioseparations [133, 139]. Good results were also obtained with dual systems consisting of CDs, such as highly sulphated-γ-CD, heptakis-(2,6-di-*O*-methyl)-β-CD and carboxymethyl-β-CD, combined each with 18-crown-6-tetracarboxylic acid [138, 139].

With respect to the separation mode, CZE and MEKC have been the most commonly used approaches for separating chiral DOAs [131, 133-137, 140]. Capillary electrochromatography was also proposed on one occasion as well, specifically for the enantiomeric separation of several synthetic cathinones using a chiral stationary phase based on amylose *tris*(5-chloro-2-methylphenylcarbamate) [146]. However, its application might be limited because it requires modifications of the stationary phase that often involve time-consuming procedures.

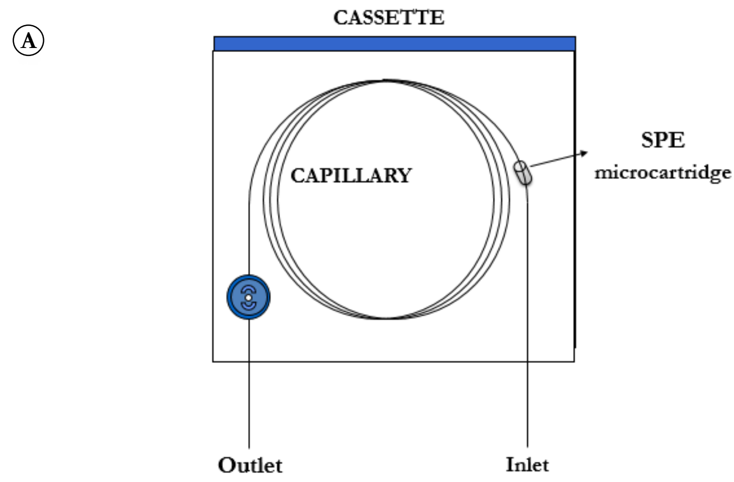
The most widely used detector was UV but other detectors, such as capacitively coupled contactless conductivity and MS, have also been employed for the same purpose [133-137, 140]. However, in the case of MS, the main drawback to using chiral selectors such as CDs is that they are not volatile, thus potentially causing source

contamination and ion suppression problems. To avoid these issues, several approaches have successfully been applied in the chiral CE-MS determination of different DOAs in seized and biological samples: (i) partial filling technique in which only a fraction of the capillary is filled with the buffer containing the chiral selector instead of fully flushing it, while the rest of the capillary is filled with the buffer without the selector. In this way, the non-volatile portion of the buffer does not enter the mass spectrometer [147]. Such an approach has recently been applied for the chiral separation of 12 synthetic cathinones in seized samples using 0.6% (w/v) highly sulphated-γ-CD in a 50 mM phosphate buffer [143]. The method provided high resolution separation and high analytical precision with RSDs of the migration time less than 1% in both intra- and inter-day experiments; (ii) Using an interface that consists of a 20 μm i.d. capillary running at a flow rate of 10 nL/min, referred to as low-flow sheathless CE-MS [148]. Here, a solution of 0.125% (w/v) highly sulphated-γ-CD in 15 mM 18-crown-6-tetracarboxylic acid was favourably used as the BGE for the enantiomeric resolution of several synthetic cathinones. RSDs of the migration time less than 3% were obtained; (iii) Using chiral polymeric surfactants, also known as micelle polymers or molecular micelles. The suppressing effect of this type of surfactants in the running buffer is reduced to a minimum because molecular micelles are hard to ionize in the ion source and heavy enough to remain outside the m/z range of the mass analyser [136, 137]. For example, the polymeric chiral surfactant poly(sodium-N-undecenoxycarbonyl-L-leucinate) was satisfactorily exploited as the chiral selector in MEKC-MS for the chiral separation and determination of various ephedrine alkaloids in standards and barbiturate drugs in serum samples [149, 150], providing excellent results.

## 1.4. IN-LINE SOLID-PHASE EXTRACTION COUPLED TO CAPILLARY ELECTROPHORESIS

As stated in earlier sections, one of the most commonly cited limitations of CE is its poor sensitivity. To overcome this problem, several strategies have been proposed in the literature, among which, the integration of SPE with CE has proven to be highly useful for improving the sensitivity of CE in the analysis of different kinds of compounds. This combination enables the analytes to be adsorbed onto a solid-phase sorbent from a large volume of sample and eluted in a much smaller volume of solvent, leading to improved LODs in comparison to CE without SPE. Sample volumes can be much larger than the capillary volume, providing very high levels of preconcentration. Different strategies have been reported for the coupling between SPE and CE, namely off-line, on-line, at-line and in-line [151-157]. Since the present Doctoral Thesis focuses on the development of analytical methods based on the in-line coupling between SPE and CE (in-line SPE-CE), this approach will be discussed in more detail here. Some authors in the field use the term on-line when referring to in-line SPE-CE [158-161].

In the most typical configuration for in-line SPE-CE, an extraction microcartridge, which contains a solid phase sorbent for retaining the target analytes, is placed as a short column near the injection (inlet) end of the separation capillary (Figure 15A), also known as a packed bed column [151-157]. Therefore, extraction, preconcentration, elution, and separation of the target analytes are carried out in the same capillary without the need for further sample handling, significantly simplifying the sample preparation procedure and enabling easy automation without CE instrument modification. To do so, as illustrated in Figure 15B, after the SPE microcartridge is conditioned and equilibrated with an appropriate solution, a large volume of sample is hydrodynamically injected (μL) (a). In this step, the analytes of interest interact with the SPE sorbent, while the impurities elute. A sample clean-up is then performed with BGE solution (b). This step allows the removal of untrapped molecules and to ensure adequate starting conditions for the electrophoretic separation. Later, a small volume of a solvent (nL) is introduced to desorb the analytes of interest (c). Afterwards, a pushing step by introducing BGE is carried out for moving the elution solvent out of the SPE microcartridge to elute the analytes from the SPE sorbent before the CE separation (d). Lastly, a selected voltage is applied and the electrophoretic separation of the target analytes begins (e).



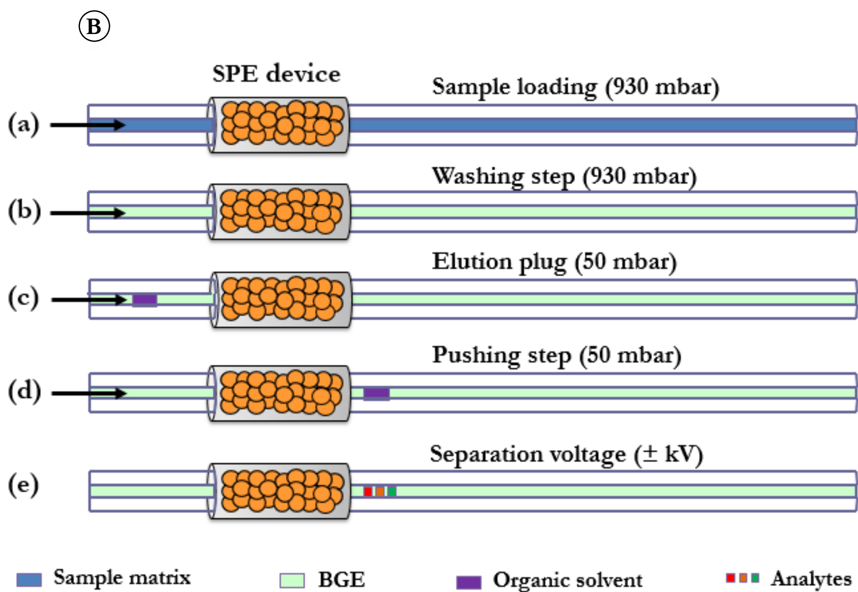




**Figure 15.** Schematic representation of (A) the in-line coupling of SPE to CE in the cassette; (B) the different steps involved in in-line SPE-CE.

There are two common approaches for the construction of a packed bed column, which are depicted in Figure 16. The first setup consists of placing the SPE material inside a small piece of capillary and two frits at either side of the packed bed to prevent the sorbent particles escaping and blocking the separation capillary (Figure 16A). This

design is usually used with SPE particles with a diameter smaller than the i.d. of the CE capillary. The second setup consists of packing a small piece of capillary with SPE particles with a size larger than the i.d. of the CE capillary. In this way, the separation capillary acts as a barrier to keep the particles in place, avoiding the use of frits and problems such as backpressure, irreproducible electroosmotic flow, bubble formation and even disruption of the separation that may occur if the frits are improperly installed (Figure 16B). Over the past six years (2011-2017), both designs have been widely applied for the preconcentration of peptides, proteins and drugs, providing satisfactory results, as shown in Table 13 [160-166] and Table 14 [158, 159, 167-178].

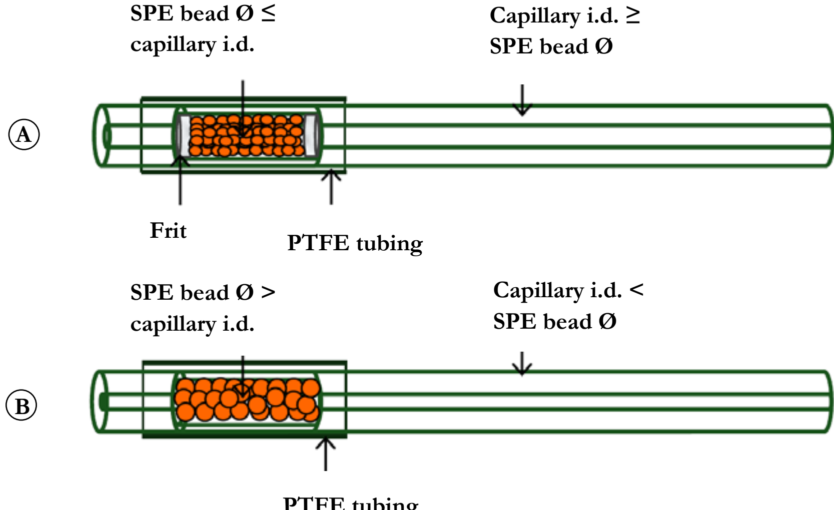



**Figure 16.** Schematic representation of the two common packed bed column designs. (A) Packed bed SPE sorbent positioned between two capillaries inside an analyte concentrator and retained by two frits; (B) Packed bed SPE sorbent positioned between two capillaries inside an analyte concentrator without frits.

As can be seen from Table 13, the frit packed bed columns proposed in the literature have been explored using sorbent materials with different selectivities, such as $C_{18}$ (reversed-phase) [160, 162, 163], immunoaffinity (IA) [164-166] and immobilized metal affinity chromatography [161]. For example, $C_{18}$-SPE-CE-MS proved to be suitable for improving sensitivity in CE for the analysis of β-carboline alkaloids of the harmala group in algae [160], achieving LOQs in the range of 0.008-0.170 ng/mL, which represented an improvement of up to 1,000 times compared to standard CE.

**Table 13.** Frit packed bed designs reported over the past six years (2011-2017).

| *Analyte* | *Sample* | *Sample pretreatment* | *SPE sorbent* | *Detection* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|---|---|
| Harmala alkaloids | *Undaria pinnatifida* algae | Incubation with MeOH; Evaporation/ reconstitution | $C_{18}$ | ESI-MS (QTOF) | 0.002-0.077 [a] | [160] |
| DynA, End1, Met | Plasma | Precipitation with ACN followed by centrifugal filtration | $C_{18}$ | ESI-MS (QTOF) | 0.1 [b] | [162] |
| DynA, End1, Met | Plasma | Precipitation with ACN followed by centrifugal filtration | $C_{18}$ | UV | 4-7 [a] (estimated) | [163] |
| End1, End2 | Plasma | Precipitation with ACN followed by centrifugal filtration | IA sorbent with intact IgG | ESI-MS (QTOF) | 100 [b] | [164] |
| End1, End2 | Plasma | Precipitation with ACN followed by centrifugal filtration | IA sorbent with Fab fragments | ESI-MS (QTOF) | 1-50 [b] | [165] |
| TTR | Serum | Double precipitation with 5% (v/v) of phenol | IA sorbent with Fab fragments | ESI-MS (QTOF) | 500 [a] | [166] |
| Amyloid β-protein (Aβ) | – | – | IMAC chelating sorbent | ESI-MS (IT) | 100-500 [a] | [161] |

See Appendix I for the key to abbreviations.
[a] Standard samples.
[b] Real samples.

**Table 14.** Fritless packed bed designs reported over the past six years (2011-2017).

| *Analyte* | *Sample* | *Sample pretreatment* | *SPE sorbent* | *Detection* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|---|---|
| DynA, End1, Met | Cerebrospinal fluid | Precipitation with ACN followed by centrifugal filtration | $C_{18}$ | ESI-MS (QTOF) | 1.0-2.5 [b] | [167] |
| DynA, End1, Met | – | – | $C_{18}$ | Sheathless ESI-MS (QTOF) | 0.002 [a] | [168] |
| Four UV filters | River water | Off-line SPE (Oasis MCX) | Oasis MCX | ESI-MS (QTOF) | 0.01-0.05 [a] | [169] |
| Five pharma-ceuticals | River water | – | Oasis HLB | UV (214 nm) | 0.19-1.00 [b] | [170] |

**Table 14.** (Continued).

| *Analyte* | *Sample* | *Sample treatment* | *SPE sorbent* | *Detection* | *LOD (ng/mL)* | *Ref.* |
|---|---|---|---|---|---|---|
| COD, EDDP, HCOD | – | – | Oasis HLB | ESI-MS (Ion trap) | 0.0002-0.0240 [a] | [171] |
| 6-AM, COC, COD, EDDP | Tap and river water | – | Oasis HLB | UV (200 nm) | 0.07-0.27 [b] | [172] |
| Three barbiturates | Urine | LLE with EtAc/hexane (40:60, v/v); Evaporation/ reconstitution | Oasis HLB | UV (214 nm) | 5-60 [b] | [173] |
| 6-AM, COD, DHCOD, EDDP | Urine | Dilution 1:1 with water | Oasis HLB | ESI-MS (IT) | 0.013-0.210 [b] | [174] |
| Three organic sulfonates | – | – | StrataX/XL-AW | UV (190, 224 nm) | 0.3-2.0 [a] | [175] |
| Eight veterinary quinolones | Milk | – | SupelMIP-SPE quinolones | ESI-MS (IT) | 3,800-4,700 ng/kg [b] | [176] |
| BP, PB, MB, BA | – | – | $C_{18}$ magnetic particles | UV | – | [177] |
| AGP | Serum | Acidification with 1 M TCA followed by centrifugation | Tosyl-activated magnetic particles | UV (214) | – | [158] |
| TTR | Serum | Immunoprecipita-tion followed by centrifugal filtration | AffiAmino Ultrarapid Agarose magnetic particles | ESI-MS (QTOF) | 1,000 [a] | [159] |
| β-lg, α-lac | Milk | Acidification with 1 M HCl followed by centrifugation and dilution | Tosyl-activated magnetic particles | MALDI-MS (TOF) | 20 [b] (β-lg); 30 [b] (α-lac) | [178] |

See Appendix I for the key to abbreviations.

[a] Standard samples.

[b] Real samples.

In another study, $C_{18}$-SPE-CE-MS was successfully combined with transient isotachophoresis, which is an electrophoretic preconcentration technique, for peptide analysis in plasma samples [162]. To do so, the authors injected the leading and terminating electrolytes before and after the SPE eluate, respectively, resulting in a very simple strategy. Under the optimal conditions in plasma, LODs as low as 0.1 ng/mL were attained, which meant a 10-fold improvement with regard to the LODs obtained by in-line SPE-CE without t-ITP (1 ng/mL).

To attempt to further enhance sensitivity, Tascon *et al.* [163] recently presented a system to evaluate the effect of temperature (between 5°C and 90ºC) on the performance of $C_{18}$-SPE-CE for peptide analysis using a mini laboratory-made thermostatic device placed inside the cassette that enabled the temperature of the in-line SPE microcartridge to be controlled and rapidly switched. The preliminary results revealed that the signal/noise ratio was dependent on the microcartridge temperature during sample loading and peaked at 60ºC. At this temperature, the authors estimated LODs of 4-7 ng/mL for standard samples, which were only slightly lower than the LODs previously reported by the same research group for standard mixtures of the same peptides in $C_{18}$ sorbent at 25ºC (10 ng/mL) [179] (paper not included in Table 13).

Medina-Casanellas and co-workers used two different IA sorbents (one with intact immunoglobin G and the second with Fab fragments) for the analysis of two opioid peptides in human plasma [164, 165] and serum transthyretin in serum [166], reaching up to 100-fold improvements in sensitivity compared to conventional hydrodynamic injection. Working with the IA sorbent with Fab fragments enabled better LODs of Endomorphin-1 and Endomorphin-2 in plasma samples (1 and 50 ng/mL, respectively) [165] in comparison with the LODs obtained for the same compounds using the IA sorbent with the antibody intact (100 ng/mL) [164]. The same research group also evaluated several commercial metal affinity sorbents for the analysis of two small peptide fragments of the amyloid β-protein (Aβ) and reported that a nickel metal ion sorbent based on nitrilotriacetic acid as a chelating agent could be useful for the preconcentration of the molecules examined, although very low recoveries of around 15% were obtained [161].

In the case of the fritless packed bed columns proposed in the literature (Table 14), the most commonly used SPE sorbents are reversed-phase, such as Oasis HLB [170-174] and $C_{18}$ [167, 168]. To provide different selectivity, other sorbents have also been

investigated, such as mixed-mode (Oasis MCX and Strata X/XL-AW) [169, 175] and molecularly imprinted polymer (MIP) [176]. In particular, the research group of Borrull and colleagues demonstrated the potential of Oasis HLB-SPE-CE for determining low levels of DOAs [172-174] and pharmaceutical compounds [170] in biological and environmental samples, providing LOQs between 0.08 and 100.00 ng/mL, with sensitivity enhancement factors ranging from 170 to 5,900 when compared with the normal hydrodynamic injection.

Meanwhile, Medina-Casanellas *et al.* [167] demonstrated the suitability of in-line $C_{18}$-SPE-CE-MS for improving sensitivity in the analysis of opioid peptides in cerebrospinal fluid, achieving LODs in the range of 1.0-2.5 ng/mL, which represented an improvement of two orders of magnitude when compared to standard CE (LOD of 100 ng/mL). In another study by the same authors, $C_{18}$-SPE-CE was combined with sheathless CE-MS using a prototype porous-tip capillary for nano-ESI for highly sensitive analysis of the same compounds (opioid peptides) [168]. Using this approach, the sensitivity enhancement was up to 5,000-fold compared to sheathless CE-MS without in-line preconcentration (LODs of 5-10 ng/mL), resulting in LODs as low as 0.002 ng/mL, demonstrating once more the extraordinary capability of in-line SPE-CE for improving LODs.

Moreno-González and co-workers, meanwhile, used a commercial MIP (SupelMIP SPE-Quinolones) as the sorbent for the construction of an in-line SPE-CE system for the determination of regulated veterinary quinolones in bovine milk samples and achieved LODs around 30 times lower than the maximum regulatory limit for these analytes. Satisfactory recoveries were obtained in the range of 70-102% [176].

Recently, Neusüß's group investigated the benefits of an arrangement of SPE particles in a fritless in-line SPE-CE setup different from that described above [175]. The arrangement proposed consists of placing a 4-mm section of 100 μm i.d. capillary filled with a string of 90 μm of Strata mixed-mode material (reversed-phase and weak anion exchange) between the inlet and separation capillaries of 50 μm i.d. Compared to the fritless packed bead configurations described above, in this work, the i.d. of the particles is much larger than the i.d. of the inlet and separation capillaries. According to the authors, this feature would contribute towards the prevention of clogging or the formation of hollow spaces inside an SPE column, since the conventional setups involve a risk of wedged beads due to the i.d. of the particles barely being larger than the i.d. of the inlet and separation capillaries. For aromatic sulphonic acids and 8-

aminopyrene-1,3,6-trisulphonic acid-labeled glycans, enrichment factors of 450-1,000-fold were achieved using the developed in-line SPE-CE method compared to standard CE, resulting in LODs in the low nanomolar range.

A simpler way to create a fritless packed bed column is to use magnetic particles held in place with a magnetic field. This consists of coating the sorbent material onto magnetic particles that may easily be manipulated inside a CE capillary simply by using external magnets located inside the cassette near the inlet end of the separation capillary. Thus, rather than holding the SPE material in a separate capillary, it can be introduced directly into the separation capillary and then easily pumped out without needing to remove the cassette containing the capillary with the analyte concentrator from the instrument, as is required when using conventional in-line SPE.

Tennico and Remcho [177] successfully synthesized silica-coated iron oxide particles functionalized with $C_{18}$ and used them as reversed-phase sorbents for the in-line extraction of several parabens and nonsteroidal anti-inflammatory drugs prior to separation by CE. To do so, the functionalized magnetic particles were added to the sample, mixed, and then injected into the CE system and retained at the inlet end of the capillary using permanent magnets placed inside the cassette. It should be noted, however, that only a partial automation of the extraction process inside the CE capillary was achieved, since the magnetic extraction was performed outside the system. Figure 17 shows a schematic drawing of the mechanism for performing the in-line magnetic extraction with CE developed. As can be seen from the figure, the steps are similar to those in conventional in-line SPE-CE methods. No LODs were given.

To date, fully automated approaches using magnetic particles in-line with CE have only been implemented for IA purification procedures, using magnetic particles coated with appropriate antibodies immobilized inside the CE capillary as immunosupport for the extraction and isolation of different proteins prior to separation by CE [158, 159, 178]. For example, Peró-Gascón *et al.* [159] satisfactorily developed an in-line IA-SPE-CE-MS method using AffiAmino Ultrarapid Agarose™ magnetic particles for the analysis of transthyretin in serum. Under the optimized conditions with standards, the LOD obtained was 25 times lower (1,000 ng/mL) than in CE-MS (25,000 ng/mL). Gasilova and colleagues used tosyl-activated magnetic particles for two major milk whey proteins immunocaptured inside a CE capillary [178]. The IA-SPE-CE method was combined with a transient isotachophoresis step

as additional sample preconcentration induced by the separation medium with one stacker as the leading electrolyte (ammonium acetate) and the coions of the elution solution (acetic acid) being the terminating electrolyte, providing an LOD of about 550 ng/mL for both whey proteins. To further improve the sensitivity, the IA-SPE-CE method was hyphenated with MALDI-TOF-MS, achieving LODs of around 20-30 ng/mL.

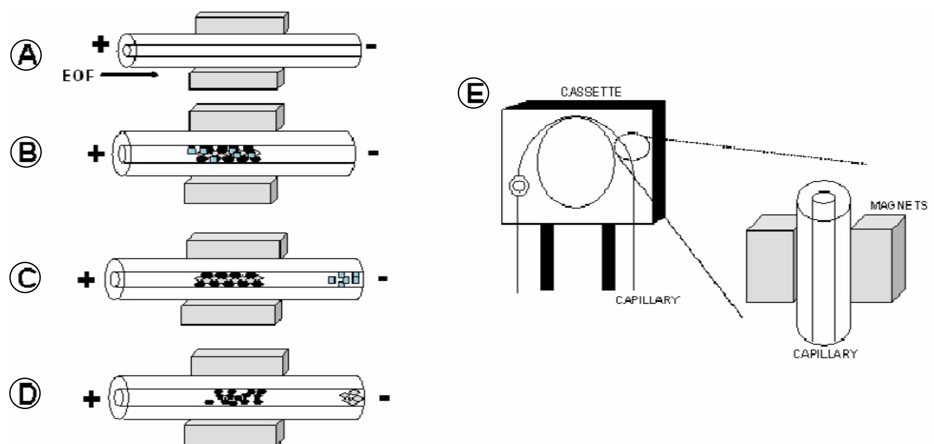



**Figure 17.** Schematic illustration of the mechanism for performing inline magnetic extraction with capillary electrophoresis. (A) Conditioning step: capillary is conditioned, neodymium iron boron permanent magnets are placed around the capillary. (B) Sample loading: sample mixtures containing magnetic particles are introduced into the capillary and are retained by the magnets. (C) Washing step: analytes of interests will interact with the sorbents, while interfering components are eluted. (D) Elution step: retained analytes are eluted by applying a stronger eluent. (E) A diagram showing how the magnets sandwiched the capillary in the cassette. Reproduced from [177] with the permission of WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, copyright 2010.

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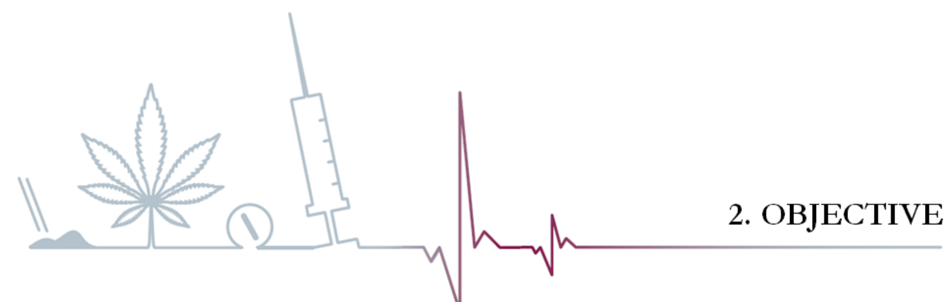

# 2. OBJECTIVE

The objective of the present Doctoral Thesis is the development and application of sensitive and environmentally friendly analytical methods based on the use of in-line SPE in combination with CE-UV. The main purpose is to be able to determine quantitatively DOAs in biological samples for application in therapeutic, legal and forensic settings, such as workplace safety programs, anti-doping monitoring in sport, crime scene investigations or rehabilitation programs.

# 3. EXPERIMENTAL, RESULTS AND DISCUSSION

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As mentioned in the introduction, in-line SPE-CE represents a powerful approach for improving CE sensitivity. For this reason, continuous evaluation and application of this methodology is an active research topic nowadays. According to a recent review by Ramautar *et al.* [1], between July 2013 and June 2015, about 20 scientific papers focusing on in-line coupled SPE-CE systems have been reported in the literature. Of these works, only one deals with the establishment of an in-line SPE-CE strategy for the determination of DOAs in biological samples [2]. Within this framework, the purpose of this Doctoral Thesis was to achieve a more in-depth exploration and expand the use of this technique in the field of DOAs testing. Therefore, four inexpensive, green and easily performed analytical methods were developed for the identification and quantification of several DOAs in human biological samples by means of the in-line SPE-CE-UV.

The present Doctoral Thesis has been developed in the research group of Chromatography and Environmental Applications at the Universitat Rovira i Virgili, which has extensive experience working with in-line SPE-CE to decrease the detection limits of CE for the determination of analytes such as pharmaceuticals, personal care products and DOAs in environmental samples [3-7]. To a much lesser extent, the group has also worked on the development of strategies based on in-line SPE-CE to be used in the analysis of DOAs in biological samples, particularly urine [2, 8]. Therefore, this Thesis aims to increase current knowledge regarding the applicability of in-line SPE-CE for the determination of DOAs in biological samples, such as hair and urine.

This chapter contains the experimental part of the research, the results, and discussion of the main findings obtained from the different studies developed over the course of this Doctoral Thesis, which are classified into two sections. Each section includes a brief introduction to summarize the background of the research, the results in journal paper format and the discussion of the most relevant findings at the end of each section. These results have already been published in different international scientific journals. A list of all the papers derived from the research project considered in this dissertation is included in Appendix III.

**The first section** covers the development of three novel analytical methods for the determination of various DOAs in hair samples. In all cases, a 2-mm segment of capillary of 150 μm i.d. packed with 60 μm particle size Oasis HLB (reversed-phase) was used for the construction of the analyte concentrator microcartridge. The first

method focused on the determination of cocaine and its major metabolite benzoylecgonine in hair samples collected from drug abusers. To extract the analytes from the hair matrix, an overnight acidic digestion procedure was combined with the in-line SPE-CE system. The second method focused on the determination of the DOAs considered in our previous research, plus the opiates 6-acetylmorphine, codeine, morphine, and methadone, in hair samples collected from drug abusers. Since methadone exists as a chiral molecule, a suitable, previously optimized CD was added to the CE electrolyte to achieve the resolution of its enantiomers. To extract the analytes from the hair matrix, in this study, a procedure based on pressurized liquid extraction (PLE) was developed and combined with the in-line SPE-CE system. Finally, the third method involved the development of a new strategy based on the in-line coupling between SPE and CD-modified-CE with a previous sample pretreatment procedure based on PLE for the chiral determination in hair samples of two novel psychoactive substances that have quickly gained popularity among young people and teenagers recently, namely 3,4-methylenedioxypyrovalerone and mephedrone.

**The second section** describes the preparation of silica-coated $Fe_3O_4$ particles functionalized with $C_{18}$ groups and their subsequent application as an SPE sorbent for the construction of an in-line analyte concentrator in CE for detecting DOAs in urine samples. The approach established was satisfactorily applied to the analysis of DOAs in urine samples obtained from drug abusers that were following a drug rehabilitation program with a simple sample pretreatment protocol based on LLE. This research work was carried out in collaboration with Professor Christian Neusüß of the Chemistry Department of the Aalen University (Germany), during a European placement that took place during the development of this Thesis.

The studies reported in this Doctoral Thesis were financially supported by the General Research Directorate of the Spanish Ministry of Science and Technology, projects CTQ2011-24179, CTQ2014-52617 and by the *Generalitat de Catalunya, Departament d'Innovació, Universitats i Empreses*, project 2009 SGR 223.

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## 3.1. HAIR TESTING FOR DRUGS OF ABUSE

As shown in Section 1.2.1., which focuses on hair drug testing, as well as the analytical techniques currently used, such as GC and LC, CE has also successfully been applied to determine DOAs and their metabolites in hair samples. However, it is important to note that all the existing CE-based methods for this purpose involve off-line LLE for the matrix clean-up and preconcentration of the target compounds. This fact may be considered as a drawback of these approaches because, as is well known, LLE techniques have several shortcomings (e.g., time-consuming operations, difficult automation, need for relatively large organic solvents volumes, etc.) [1], making the methodologies proposed in the literature unsuitable for routine DOAs testing. This scenario prompted us to continue working in this field and, as such, the research presented in this section was carried out in response to the need for developing analytical methods based on greener strategies with minimal sample-handling, suitable for the determination of DOAs in hair samples, such as the in-line coupling between SPE and CE.

**In the first study**, the suitability of a method based on in-line SPE-CE with UV detection was evaluated for the determination of cocaine and its major metabolite, benzoylecgonine, in hair samples. Five commercially available SPE sorbents were examined to select the most appropriate for our purpose. The research group in which I have carried out my Doctoral Thesis has previously studied the determination of DOAs in river, tap water and urine samples by in-line SPE-CE-UV using Oasis HLB, a polymeric reversed-phase sorbent, as the sorbent for the construction of the SPE microcartridge, achieving LODs in the range of 0.07-60 ng/mL in real samples [2, 3]. Motivated by the satisfactory results obtained, Oasis HLB was one of the sorbents investigated with the aim of assessing its potential when dealing with the in-line preconcentration of DOAs extracted from hair samples.

However, it is now well known that the major drawback of the reversed-phase materials is their limited selectivity [4]. Several attempts were made to improve the in-line SPE-CE method's selectivity for DOAs testing by exploring various SPE sorbents with chemical properties different from those of Oasis HLB, particularly Oasis MCX, Bond Elut Plexa PCX, LiChrolut TSC and LiChrolut SCX, all of which are cation exchangers. This choice was made in view of the fact that cocaine and benzoylecgonine are basic and, at low pH values, would be retained favourably on these cationic exchangers, thereby contributing to greater selectivity.

Once the SPE sorbent had been selected, different parameters affecting in-line SPE-CE were carefully optimized to get the desired analytical performance to achieve the maximum sensitivity enhancement, namely sample pH, the elution volume and sample loading time.

As discussed in Section 1.2.1., a variety of procedures can be found in the literature for the extraction of DOAs from the hair matrix (such as acidic or alkaline digestion, solvent extraction, enzymatic hydrolysis and incubation with various buffer systems, being mostly held overnight). Of these methods, an approach based on an overnight digestion with dilute hydrochloric acid was used to extract cocaine and benzoylecgonine prior to in-line SPE-CE-UV analysis. An acidic digestion was chosen since the extraction solutions resulting from this procedure could be directly injected into the previously established in-line SPE-CE system without the need for evaporation and reconstitution steps, allowing the two techniques to be easily combined.

Encouraged by the promising results obtained in our first work, our efforts then focused on exploring new and effective strategies that could enable the extraction process to be faster compared with the conventional extraction methods stated above, which generally involve periods of 16-20 hours (see Section 1.2.1.). Recently, an Italian research group showed the potential of PLE for the isolation of different DOAs from hair (e.g., amphetamines, opiates, synthetic cathinones, etc.) followed by off-line SPE for sample clean-up and LC-MS/MS analysis, completing the extraction process within just 15 minutes, which represented a significant achievement in the field of hair drug testing [5, 6].

With this in mind, **in the second study** included in this section, a further step was taken to establish an improved PLE procedure for the extraction of a group of DOAs, including 6-acetylmorphine, benzoylecgonine, cocaine, codeine, morphine and methadone from hair suitable for combination with in-line SPE-CE. The PLE process was optimized testing the extraction solvent composition, temperature and static time. Special attention was paid to obtaining hair extracts compatible with the in-line SPE-CE method proposed, as well as developing an environmentally friendly approach.

Another important aim of this study was to provide a CE strategy capable of resolving chiral compounds, since methadone occurs as a chiral compound. As stated in Section 1.3.2., the enantiomers of a given drug might have different pharmacological and

toxicological effects and, consequently, the development of analytical methods that could achieve the identification and quantification of each of them in biological samples is of the utmost importance. CE using CDs represents a very powerful analytical tool for separating chiral DOAs (see Section 1.3.2.).

In response to this scenario, our intention was to develop an efficient CE method for simultaneously separating chiral and non-chiral analytes using a BGE with CD. The CD type and concentration were evaluated to find out the most suitable electrophoretic medium, taking into consideration that, in in-line SPE-CE systems, the sorbent is fully integrated into the capillary and, therefore, both the SPE-CE microcartridge and CD selected should be highly compatible. This fact represented an important requirement for obtaining successful results. To the best of our knowledge, this was the first experience reported in the literature dealing with the use of CDs in combination with an in-line SPE-CE-UV system.

Oasis HLB was the sorbent used for the construction of the concentrator device because of the good results obtained for cocaine and benzoylecgonine in our previous research work. The in-line SPE-CE performance was optimized testing the elution volume and sample loading time.

Recently, great interest has focused on synthetic cathinones since their consumption has increased exponentially. All synthetic cathinones exist as chiral molecules. Within this context, knowledge of the enantiomeric composition of these analytes in biological samples could provide valuable assistance in interpreting drug testing results. However, the literature concerning the chiral analysis of these DOAs in biological samples is very scarce. According to a very recent review [7], there are only three reports related to this topic developed for urine and plasma samples, and none focused on hair samples.

Once familiarized with the combination of PLE and in-line SPE-CD-modified-CE for detecting DOAs in hair samples, **in the third study** presented in this section, a novel method based on this combination was developed for the chiral determination in hair of three synthetic cathinones, namely mephedrone and one of its metabolites, 4-methylephedrine, and 3,4-methylenedioxypyrovalerone. Mephedrone and 3,4-methylenedioxypyrovalerone were investigated because they are two of the most frequently reported ingredients in bath salts, the name under which these DOAs are often marketed [8, 9]. As in our previous study, our efforts focused on assuring that

the extraction, preconcentration and chiral separation procedures used would be compatible together. To select the most appropriate PLE conditions for the extraction of the target synthetic cathinones from the hair matrix, the solvent composition, temperature and static time were examined. The effect of the CD type and concentration on the resolution of the target compounds was carefully studied. The sorbent used to construct the in-line SPE-CE microcartridge was Oasis HLB as, in the research works introduced above, it was demonstrated that this sorbent is a suitable SPE material for enriching DOAs. Optimization of the process of in-line preconcentration involved the evaluation of the elution volume and sample loading time.

The three analytical methods established were validated in accordance with the Scientific Working Group for Forensic Toxicology [10] and Society of Toxicological and Forensic Chemistry [11] guidelines. Linearity, repeatability, reproducibility, LOD, LOQ and relative recoveries were evaluated using pooled drug-free hair samples spiked with the studied analytes.

Finally, the validated analytical methods in the first and second study were applied to the hair of drug abusers that were undergoing a drug detoxification program. It is usually stated that head hair grows at a rate of approximately 1 cm per month and can be evaluated along the length of the hair shaft to provide a profile of use and/or exposure to a drug over time [12]. Segmental hair analysis was performed in both cases to determine the target individuals' compliance with the therapy. To this end, the samples were divided into 1.5 cm segments of hair, starting from the site of cutting to the end of the strands collected.

The results of the three studies were published in the following scientific journals: Bioanalysis 7 (2015) 437-447, Journal of Pharmaceutical and Biomedical Analysis 131 (2016) 420-428 and Electrophoresis 37 (2016) 2352-2362, and are presented in the following sections.

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### *3.1.3. Determination of cocaine in abuser hairs by CE: monitoring compliance to a detoxification program*

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# DETERMINATION OF COCAINE IN ABUSER HAIRS BY CE: MONITORING COMPLANCE TO A DETOXIFICATION PROGRAM

**Tatiana Baciu, Francesc Borrull, Marta Calull* and Carme Aguilar**

Department of Analytical Chemistry and Organic Chemistry, Rovira i Virgili University, Campus Sescelades, Marcel·lí Domingo, 1, 43007 Tarragona, Spain

## Abstract

**Background:** Segmental hair analysis was performed to verify the cocaine withdrawal and compliance to the therapy of four cocaine abusers that were following a drug detoxification program. **Results/methodology:** Cocaine and its major metabolite, benzoylecgonine, were preconcentrated and determined by in-line SPE and CE with prior isolation of the analytes from the hair matrix by an overnight acidic incubation procedure. The LODs obtained for hair samples were 0.02 ng/mg for cocaine and 0.1 ng/mg for benzoylecgonine. **Conclusion:** Our results showed that the established method, in conjunction with a segmental hair analysis, is suitable for determining drug abuse histories, being very useful in forensic toxicological laboratories as well as in rehabilitation and addiction treatment programs.

*Key terms: cocaine (an addictive drug derived from coca or prepared synthetically used as an illegal stimulant)/benzoylecgonine (cocaine metabolite most often identified in toxicology)/hair analysis (chemical analysis of hair in order to find possible sign of exposure and/or intake of a substance)/CE (analytical technique that separates charged molecules based on their electrophoretic mobility when a voltage is applied)/SPE (an extraction method that uses a solid phase and a liquid phase to isolate the target compounds from a solution. Usually, it is used for cleaning up samples and for the preconcentration of analytes before instrumental analysis)*

Cocaine (COC), an alkaloid derived from the *Erythroxylum coca* plant, is known to be a potent and addictive stimulant which can alter the brain. For hundreds of years, it has been used as a local anesthetic during surgeries. However, in recent years, COC abuse has become epidemic. Once ingested, it is primarily hydrolyzed in the body to benzoylecgonine (BZE) and ecgonine methyl ester, but it is also metabolized to norcocaine and several other metabolites [1]. As a result, COC abuse can be proven by the detection of its metabolites, which suggest ingestion of the drug [2]. Nowadays, these analytes are determined in various biological matrices, including urine, blood, oral fluids and hair [3].

Following up prescribed and non-prescribed drug use, and on the contrary, drug abstinence, during drug treatment programs is important for the assessment of compliance and success of the treatment. In comparison to the more traditional biological matrices, such as blood and urine, hair offers particular advantages that have already been discussed elsewhere [4]. Generally, human adult scalp hair grows on average 1 cm per month and this means that each centimeter of hair records about 1 month of the individual's use or exposure to a drug. Therefore, 1 cm length segmental hair analysis can be used to estimate the monthly consumption of illicit drugs [5, 6]. Several papers have been focused on segmental hair analysis for the determination of opiates [7], amphetamines [6], benzodiazepines [8], cannabinoids [9] and cocaine [10].

In recent years, several analytical procedures have been reported in the scientific literature for determining a broad range of drugs and their metabolites in hair samples, most of them based on gas or liquid chromatography as the determination technique [4]. Besides these techniques, capillary electrophoresis (CE) has also been successfully applied for this purpose [11–13]. Typically, the capillary electrophoretic methods for drug testing in hair are based on the use of liquid-liquid extraction (LLE) for the matrix clean-up and preconcentration of the target compounds. This fact may be considered as a drawback due to the large volume of organic solvents used. In line with the environmentally friendly procedures tendency, further research is needed and should be focused on the development of greener strategies, such as combinations between CE and microextraction techniques.

With this in mind, the aim of this study was to establish a cheap, simple and environmentally friendly method

to simultaneously determine COC and its major metabolite, BZE, in hair by means of the in-line coupling between solid phase extraction (SPE) and CE-DAD. Since SPE is in-line coupled to CE-DAD, the global analysis time and workload may be reduced. The method was applied to hair samples of COC abusers that were following a drug rehabilitation program on the basis of segmental analysis in order to determine their compliance to the therapy. To the extent of our knowledge, this is the first application of in-line SPE–CE-DAD for the analysis of these illicit substances in a complex matrix such as human hair.

## Experimental

### Reagents & standards

All reagents used were of analytical-reagent grade. Ultrapure reagent water obtained from a water purification system (Millipore, Bedford, MA, USA) was used throughout this work. COC and BZE were purchased from Cerilliant (TX, USA). Methanol (MeOH) and dichloromethane (DCM) were acquired from VWR (PA, USA). Formic acid (HCOOH), phosphoric acid ($H_3PO_4$), hydrochloric acid 37% (HCl), sodium phosphate monobasic ($NaH_2PO_4$), ammonium hydroxide 28% ($NH_4OH$) and acetic acid ($CH_3COOH$) were acquired from Sigma-Aldrich (MO, USA). Different SPE sorbents with an average particle size of 60 µm were used: Oasis HLB and Oasis MCX from Waters (Mildford, MA, USA), Bond Elut Plexa PCX from Agilent Technologies (Waldbronn, Germany) and LiChrolut TSC and LiChrolut SCX from Merck (Darmstadt, Germany).

Stock standard solutions of 100 mg/L for each compound were prepared in MeOH and stored at -18°C. From these standard solutions, working standard solutions of the mixture of all the compounds at a concentration of 0.1 mg/L were prepared weekly by diluting in Milli-Q water. The working solutions with a lower concentration were prepared daily by appropriate dilution of the mixed solution with ultrapure water.

### Instrumentation

The electrophoretic system was $HP^{3D}$ CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with an UV DAD. The analytes were detected at 200 nm. Bare fused-silica capillaries with an internal diameter (i.d.) of 50 and 150 µm were purchased from Polymicro Technologies (AZ, USA). The capillary chamber was set at 25°C for

all the experiments. For pH measurements, a Lab pH-meter Basic 20+ (Crison, Barcelona, Spain) was used.

**Hair samples collection**

Hair samples were provided by the CAS Tarragona Drug Addiction Monitoring and Support Centre (GIPPS Health) in Tarragona, Spain. The samples were collected from four volunteers with a past drug abuse and, at the time of sampling, they were following a drug rehabilitation program in the aforementioned center. The hair collection procedure was performed as described in a recent published review [4]. Depending on the hair length of each individual, different lengths of hair specimens were taken. As discussed, each centimeter of hair keeps the record of about one month of drug usage, so the specimens were collected in different consecutive months, starting the sampling in June 2013. Detailed data concerning subjects under study and their hair samples are presented in Table 1. Hair used to prepare blank and calibration samples was collected from the laboratory personnel following the same procedure.

**Hair sample preparation**

The strands of obtained hair were divided into several segments of about 1.5 cm long from the site of cutting to the end of the strand. These were individually decontaminated twice by shaking them in 2 ml DCM for 5 min each time by means of a vortex. After being washed, the hair segments were dried under a gentle $N_2$ stream at ambient temperature, weighed (50 mg each segment) and subsequently cut with scissors into segments of 1–2 mm. Afterwards, 2 ml of 0.1 M HCl was added and then incubated in a water bath at 45°C overnight (≈ 14 h). Following the heating period, the pH was adjusted to pH 9.5 with the addition of 2 ml of 0.1 M NaOH. The solution was then filtered through a 0.45 µm polytetrafluoro-ethylene (PTFE) filter and, lastly, it was brought to a volume of 5 ml with the addition of Milli-Q water. Finally, 1 mL of this extract was transferred to a microvial for in-line SPE–CE-DAD analysis as described below. Drug-free hair samples from laboratory volunteers were used for method validation following the same procedure described.

**Table 1.** Characteristics of the four individuals under study.

| *Subject* | *Age* | *Sex* | *Hair length* | *Hair segments of 1.5 cm* | *Substance declared used* | *Months of sampling* |
|---|---|---|---|---|---|---|
| A | 30 | M | 5 cm | Three | COC in use for 2 years; ended its intake 3 months prior to June 2013 | June and July 2013 |
| B | 40 | F | 9 cm | Six | COC in use for years; ended its intake 3 weeks prior to June 2013 | June 2013 |
| C | 40 | M | 7 cm | Four | COC in use for years; ended its intake 10 months prior to June 2013 | June and July 2013 |
| D | 30 | M | 3 cm | Two | COC in use for 15 years; ended its intake 2 months prior to June 2013 | June, July and August 2013 |

## Electrophoretic procedure

The electrophoretic separation was performed on a fused-silica capillary provided by Polymicro Technologies with a total length of 65.5 cm (57 cm effective length) with an i.d. of 50 µm and an outer diameter (o.d.) of 360 µm. The separation voltage was 30 kV (positive polarity). The background electrolyte (BGE) consisted of an aqueous solution of 55 mM $NaH_2PO_4$ and 25 mM of $H_3PO_4$ at pH 2.5. Before first use, the capillary was conditioned with 1 M NaOH for 40 min and Milli-Q water for 10 min. Before each run, the capillary was rinsed with 0.1 M NaOH for 5 min and Milli-Q water for 5 min. The capillary was then conditioned by flushing with BGE for 10 min. Between experiments, the capillary was conditioned with 0.1 M NaOH for 4 min, Milli-Q water for 4 min and running buffer for 4 min. Samples were injected using a hydrodynamic injection applying a pressure of 50 mbar for 10 s.

## Construction of the analyte concentrator

The analyte concentrator (AC) was constructed as described in previously published works [14, 15]. Briefly, the first step was to cut 2 mm of bare fused-silica capillary of 150 µm i.d. and 360 µm o.d. A good cut on the

two sides of the capillary is fundamental for achieving optimum performance of the concentrator. This short segment of capillary was hooked to a vacuum pump and the Oasis HLB sorbent was loaded into the concentrator. Sorbent particles with an average particle size of 60 μm were used. The AC was then inserted halfway into a 0.50 cm piece of PTFE tubing (Grupo Taper S.A., Madrid, Spain) with an i.d. of 0.25 mm. PTFE material can enlarge to fit the o.d. of the bare fused-silica capillary. Later, a 7.5 cm segment of bare fused-silica capillary (50 μm i.d. × 360 μm o.d.) was coupled to one side of the AC (inlet) and the CE separation capillary of 50 μm i.d. × 360 μm o.d. was coupled to the other side of the concentrator (outlet). The whole process of fabricating the AC was followed under a microscope.

## In-line SPE-CE procedure

Prior injection, the capillary with the AC was conditioned at 930 mbar with MeOH for 5 min and Milli-Q water (adjusted to pH 9.5 with 1 M NaOH) for 5 min. The in-line SPE procedure proposed in this paper for CE analysis of the studied drugs is described below. The first step involves the injection of standard solutions or samples (adjusted to pH 9.5 with 1 M NaOH) using a pressure of 930 mbar for 30 min. Sample clean-up is then performed with BGE solution by applying 930 mbar for 1 min. This step allows the removal of untrapped molecules and to ensure adequate starting conditions for the separation. Afterwards, the retained analytes were eluted by injecting a plug of MeOH with 2% of $CH_3COOH$ at 50 mbar for 20 s. The elution plug was then removed from the AC with the BGE at 50 mbar for 250 s. Lastly, a voltage of 30 kV was applied for the electrophoretic separation of the two analytes.

## Method validation

The developed analytical method for the quantitative determination of COC and BZE was evaluated in terms of linearity, repeatability, reproducibility, LOD and quantification LOQ, and relative recovery. For these experiments, the extraction and subsequent in-line SPE–CE-DAD analysis was conducted as described above.

The linearity was examined for each analyte by evaluating the coefficient of determination ($r^2$) after linear regression analysis, by analyzing 50 mg of blank hair samples spiked prior the incubation process with the drugs at concentrations between 0.06 and

6.00 ng/mg. Three replicates of each concentration level were analyzed.

LOD and LOQ values were determined by analyzing blank hair samples (50 mg) spiked with the drugs prior the incubation process. The LOD was considered as the lowest detectable concentration yielding a signal to noise ratio of three and the LOQ was taken as the lowest quantifiable analyte concentration yielding a signal to noise ratio of ten.

The repeatability and reproducibility values, expressed as the relative standard deviation (%RSD), were evaluated by injecting five replicates on the same day and on five different days, respectively. For that purpose, 50 mg of blank hair samples were spiked at a concentration of 1.00 ng/mg prior the pretreatment procedure and then analyzed as aforementioned.

For the relative recoveries determination, 50 mg of blank hair samples previously washed and cut and Milli-Q water were spiked prior their incubation at a concentration of 1.00 ng/mg and then processed as described above. The percent of relative recovery was calculated for each drug as the concentration of analyte found in the blank spiked samples divided by the concentration of analyte in the standard solutions. The mean extraction yield values were calculated from three independent analyses.

## Results and discussion

This section describes the optimization and validation process of the proposed in-line SPE–CE-DAD method for determining COC and BZE in hair samples, and the results obtained when hair strands of four drug abusers were analyzed.

## Optimization of the CZE separation

As stated in the scientific literature, COC possess a p$K_a$ of 8.6, while for BZE, the p$K_a$ values are 3.1 and 10.1 [16]. Therefore, capillary zone electrophoresis (CZE) separation is possible at pH levels lower than 3, since under these conditions, the analytes are positively ionized. Therefore, for the optimization of the separation of the studied compounds by CZE in an acidic medium, two different BGEs were selected and subsequently evaluated. This choice was made according to existing papers in which HCOOH and sodium phosphate buffers were employed to analyze several kinds of drugs [13, 16]. The studied electrolytes were 60 mM HCOOH at pH 2.5 and a buffer solution composed of 55 mM $NaH_2PO_4$ and 25 mM $H_3PO_4$ at pH

of 2.5. In terms of resolution and migration times, no significant differences were obtained between the two BGEs. However, the best results regarding sensitivity (peak height) were achieved using the phosphate buffer solution, so this was chosen for further studies.

**Preconcentration of the drugs by in-line SPE–CE-DAD**

At the beginning of the method development, the efficiency of five different SPE sorbents materials was studied to select the most appropriate for the clean-up and preconcentration of the target compounds. Hydrophilic polymeric sorbents have become increasingly popular due to their high surface area and polar functionalities in their structures. Of these, Oasis HLB, which is based on a macroporous poly(*N*-vinylpyrroli-donedivinylbenzene) copolymer, has been one of the most widely used and favourably applied for determining different drugs of abuse in biological samples, including hair, with good results [14, 17–18]. Moreover, based on the basic properties of the studied analytes, three mixed-mode (Oasis MCX, Bond Elut Plexa PCX and LiChrolut TSC) and one strong cation-exchange (LiChrolut SCX) sorbents have also been evaluated in order to improve selectivity, with Oasis MCX being the most widely applied for extracting illicit drugs, including COC and BZE, from biological samples, such as hair, providing good recoveries [19–21]. In contrast, for the remaining sorbents, as far as we know, there is no application in the field of the determination of drugs of abuse.

Besides the ability of the sorbent materials under study to retain COC and BZE, their selection was influenced by the commercial availability of the size of their particles, which had to be greater that the i.d. of the separation capillary in order to avoid that the sorbent particles escaping from the system, since a fritless design in-line SPE–CE has been constructed and subsequently used in this work. The separation capillary had an i.d. of 50 μm, so SPE sorbents with an average particle size of 60 μm were chosen.

For this study, standard solutions containing both drugs at a concentration of 100 μg/L were loaded onto the sorbent at 930 mbar for 5 min. A washing step was then carried out with the BGE solution at 930 mbar for 1 min, and, finally, for the elution step, a plug of solvent was introduced into the capillary at 50 mbar for 5 s. This plug was then driven through the SPE sorbent by means of BGE solution that was introduced at 50 mbar for 250 s. The

pushing step is needed for moving the plug of elution solvent out of the SPE device to elute the analytes from the sorbent. For that purpose, the pushing step was optimized at different times between 140 and 250 s in order to be sure that the elution plug was displaced out of the AC. The best conditions were achieved with 250 s at 50 mbar, since at lower injection times problems with current losses occurred, probably caused by the presence of a part of the elution solvent within the SPE device when the separation voltage was applied. Under these conditions, the volume of the capillary filled was around 0.293 μL, which corresponds to 15.1 cm of the capillary length (calculated using the Poiseuille equation). This was sufficient to get out the elution solvent out of the SPE sorbent.

Oasis HLB is a universal and polymeric reversed-phase sorbent with both hydrophilic and lipophilic retention characteristics. Different sample pH values have been published when this one has been used to determine drugs of abuse [14], so to ensure the most appropriate conditions for retaining both drugs included in this work, the sample pH was studied. Taking into consideration the $pK_a$ values of COC and BZE, mentioned in the previous section, sample pH levels of 2.5, 7 and 9.5 were tested. For that purpose, standard solutions of 100 μg/L were either acidified to pH 2.5 or alkalinized to pH 9.5 using 1 M HCl or 1 M NaOH, respectively. As elution solvent, MeOH containing 2% of $CH_3COOH$ was employed. In this way, COC and BZE become positively charged and poorly retained, and they can be released from the sorbent under these conditions. Higher signal response in terms of peak area was obtained when the pH of the sample was 9.5, since at this one, COC is uncharged and is retained by the strength of lipophilic interactions, while BZE is zwitterionic and is retained by the strength of hydrophilic interactions, so that their retention in the sorbent is favoured.

Oasis MCX, Bond Elut Plexa PCX, LiChrolut TSC and LiChrolut SCX are cation exchangers and in principle, their better extraction conditions are expected at pH values at which the analytes are in their cationic form. Therefore, to exploit the ion exchange retention mechanism between the sorbent and the analytes, the sample should be adjusted to a low pH, so that COC and BZE are positively charged. Thus, the sample was adjusted to pH 2.5 with 1 M HCl. As elution solvent, MeOH containing 2% of $NH_4OH$ was employed. In this way, COC becomes neutral and BZE negatively

charged, and they can be released from these sorbents under these conditions.

Higher analytical responses regarding peak area were provided using Oasis MCX and Oasis HLB. This behaviour is rather surprising because Bond Elut Plexa PCX, LiChrolut TSC and LiChrolut SCX sorbents are cation exchangers and a similar retention to that achieved with Oasis MCX would be expected. In the case of LiChrolut sorbents, an explanation could be the fact that the particles shape is irregular, leading to bad packing in the in-line SPE–CE device and, consequently, poor interaction of the analytes with the sorbent could occur, whereas in the case of the Oasis sorbents, the shape is spherical. On the other hand, when the mixed-mode and the strong cation-exchange sorbents were used, problems with current disruption in the system and lack of repeatability in subsequent CE analysis were detected, which could be attributed to the phenomena mediated by the difference between the pH of the solvent elution (MeOH with 2% $NH_4OH$) and the BGE (phosphate buffer solution at pH 2.5). In view of these preliminary results obtained, which show that Oasis HLB is the most suitable SPE sorbent, this one was selected to enrich COC and its metabolite BZE.

Once selected the SPE sorbent, the elution volume was investigated by introducing MeOH containing 2% of $CH_3COOH$ with a hydrodynamic injection for different periods of time from 5 to 40 s in order to obtain higher sensitivity in terms of peak area. The results showed that the peak area for the tested drugs increased with the increase of the elution plug time. However, over 20 s, instability in the current profile was observed, probably due to the presence of this very low conductivity plug inside the separation capillary. Therefore, 20 s was chosen as the elution time for further studies, which corresponds to 23 nL (calculated using the Poiseuille equation) of organic solvent needed for eluting both analytes. This fact makes the proposed method in this work an attractive environmentally sustainable analytical tool.

In the next step, the sample injection time was evaluated between 15 and 50 min, using a 0.5 μg/L standard solution of the two analytes at 930 mbar, since injecting large volumes of samples represents the simplest way to increase sensitivity and therefore obtain lower LOD values. The peak areas increased with the sample loading time up to 40 min, and this means that saturation of the SPE sorbent was not exceeded. For higher injection times, the analytical

response regarding peak area remained constant for both drugs. In the end, to avoid prolonging the overall analysis time too much, it was decided that a sample loading time of 30 min was a reasonable time, providing good sensitivity for both compounds. Therefore, this time was selected to validate the analytical method.

**Analysis of hair samples**

**Hair sample preparation**

An analytical method for determining drugs of abuse in hair samples should include before analysis a proper cleaning procedure in order to remove any possible external contamination [22, 23]. There are different washing strategies reported in the literature and all of them usually consist of using a solvent or a mixture of solvents, and the general purpose is that these ones should remove external impurities as fully as possible, but without extracting the drugs from the hair matrix [4]. DCM is considered to be a suitable wash solvent because it does not penetrate into the keratin matrix (often described as a 'non-swelling' solvent), in contrast to aqueous solvents or MeOH, and thus, no relevant extraction will take place during the washing procedure [24, 25]. With this in mind, in our study, the decontamination of the hair segments was performed by DCM.

The washing step is generally followed by the extraction of the drugs and their metabolites, which typically consists of releasing the drugs from the hair matrix and then an additional step to clean-up and preconcentrate the sample is required. A variety of methods are proposed in the literature for this task [4]. Methods based on alkaline digestion cannot be used for COC analysis because, under these conditions, it is hydrolyzed to its metabolite BZE. On the other hand, in most cases reported to date, enzymatic hydrolysis is not the most selected method, since it is rather expensive. MeOH has been shown to be a good extraction solvent but the solutions obtained are not compatible with the in-line SPE–CE configuration used in this study, since the sorbent is an integrated part of the analytical system and the sample flows through this one during the injection. Whereas extraction solutions resulting from acidic incubation can be directly injected into our system without the need of an additional evaporation and redissolution step, in contrast to the methanolic extractions, and thus, incubation in 0.1 M HCl was carried out overnight for isolating COC and BZE from the hair matrix.

For the clean-up and preconcentration step, several techniques can be found in the scientific literature [4]. Considering that the proposed method in this paper involves a SPE procedure, as this is coupled in-line with CE, the hair extracts were analyzed directly after the incubation process by means of the in-line SPE–CE method. Figure 1 shows the electropherograms obtained of the extracts of a blank hair sample (A) and a blank hair sample spiked with investigated compounds at a concentration of 5 ng/mg (B). After the incubation period, the hair extracts were directly analyzed under the optimum in-line SPE–CE-DAD conditions. From the figure, it can be seen that no interfering peaks at the retention time of COC and BZE were observed, and clean electropherograms with little chemical background noise were obtained. This fact shows the specificity of this method. As a result, it should be pointed out that in this work, an efficient and an inexpensive determination method employing non-sophisticated instrumentation that is easy to handle, has successfully been developed.

## Method validation

Method validation results are summarized in Table 2. Drug identification was performed considering their retention times and absorption spectra. The six-point calibration graphs were linear over the range from LOQ to 6.00 ng/mg for both drugs with $r^2$ greater than 0.99.

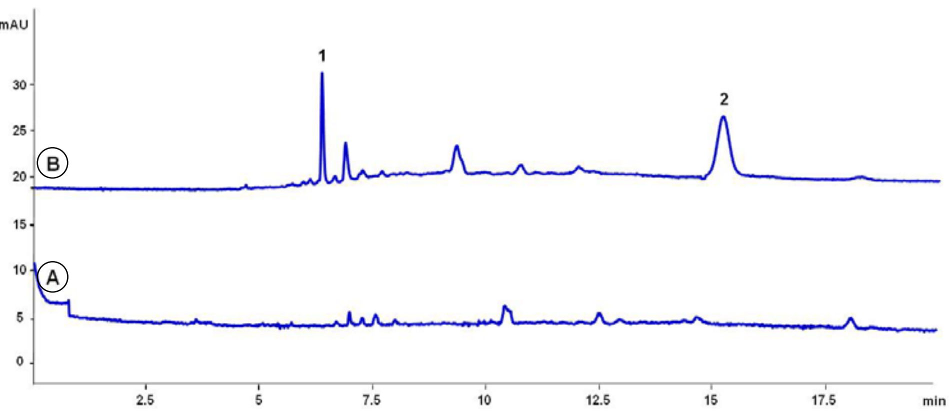


**Figure 1.** Electropherograms obtained under the optimal in-line SPE–CE-DAD conditions of: (A) an extract of a blank hair sample, and (B) an extract of a blank hair sample spiked at a concentration of 5 ng/mg. Other experimental conditions are reported in the text. Peak assignments: 1, COC and 2, BZE.

**Table 2.** Regression equations, values for reproducibility, repeatability, LODs and relative recoveries obtained for hair samples by in-line SPE-CE.

| *Validation parameters* | *COC* | *BZE* |
|---|---|---|
| Linearity (ng/mg) | 0.06–6.0 | 0.30–6.0 |
| Calibration curve † | y=59.89x+8.005 | y=97.77x+14.960 |
| $r^2$ | 0.998 | 0.993 |
| LOD (ng/mg) | 0.02 | 0.10 |
| Reproducibility (%RSD) ‡ | 7.4 | 5.7 |
| Repeatability (%RSD) § | 8.5 | 8.8 |
| Relative recoveries (%, n=3) (1.0 ng/mg) | 98 | 85 |

† y: peak area value (mAU × seconds); x: concentration (ng/mg).
‡ inter-day analysis (%, n=5) (1.0 ng/mg).
§ intra-day analysis (%, n=5) (1.0 ng/mg).
BZE: benzoylecgonine; COC: Cocaine.

LOD values were 0.02 ng/mg for COC and 0.10 ng/mg for BZE, and LOQ values were 0.06 ng/mg for COC and 0.30 ng/mg for BZE, respectively, suggesting good capacity of the proposed method for the quantification of each of the tested drug. The sensitivity achieved is comparable to the published in the scientific literature [11, 26–31]. For instance, the established method by Gottardo et al. [11], to determine different drugs in 100 mg of hair samples using overnight incubation in an acidic medium and LLE for samples preparation, and CE–MS analysis, showed results similar to those presented in this work (an LOD of 0.015 ng/mg for COC and of 0.1 ng/mg for BZE), but it should be pointed out that, in our case, 50 mg of hair specimens were employed, thus making the method more feasible for drug testing. Another study conducted on the same sample type and size as in this present paper, employing overnight incubation in an acidic medium and SPE (mixed-mode) for samples preparation, and gas chromatography (GC)–MS analysis, gave values of LOQ for COC and BZE similar to those obtained in our study (values of 0.1 and 0.2 ng/mg for COC and BZE, respectively) [31]. Taking into account the usual levels of COC and BZE reported in hair specimens from COC users (0.10–200 ng/mg) [32], the LOD and LOQ values obtained in this work allows the determination of these compounds with sufficient sensitivity in this type of sample.

As can be seen from Table 2, good intra- and inter-day precision for the in-line SPE–CE-DAD procedure was achieved for hair samples, with %RSD values lower than those obtained by Gottardo *et al.* [11]. Cordero and Paterson [31] reported lower values for COC (3.4%) and BZE (4.8%) using GC–MS as the determination technique. However, in the analysis of drugs of abuse by GC, derivatization of the compounds before instrumental analysis is a requisite and this additional step can be considered as a drawback, since it clearly lengthens the analysis time. On the other hand, relative recoveries greater than 85% were obtained, as shown in Table 2. These data are satisfactory given the complexity of hair matrix, making the proposed method proper for routine determination of the tested compounds in hair specimens.

## Hair samples from drug abusers

The validated analytical method was applied to hair samples obtained from four COC abusers (A, B, C and D) that were following a drug rehabilitation program in order to determine their COC withdrawal and compliance to the therapy. Hair samples were extracted and analyzed as described above. It is well known that segmental hair analysis can provide a map of the drug exposure over time since each centimetre of hair keeps the record of about 1 month of use or exposure of the individual to a drug [4]. With this in mind, COC and BZE concentrations were investigated in the hair segments of 1.5 cm long from the site of cutting (proximal segment) to the end (distal segment) of the strands in the hair samples collected in different months. All the quantitative results obtained are presented in Table 3.

The first sampling was in June 2013. As can be seen from Table 1, because of their different total lengths, the hair strands from the different volunteers were segmented into more or less sections; a total of 15 sections were thus analyzed. Eleven of them were positive for both illicit drugs, in the concentrations range between 0.50 and 5.10 ng/mg. The Society of Hair Testing recommends the presence of COC at a concentration above the cut-off of 0.5 ng/mg and at least one metabolite, mainly BZE, at a concentration of 0.05 ng/mg or higher, to confirm the consumption of the drug and to rule out false-positive results [2, 19]. The four hair segments that were tested negative for COC, corresponded to the volunteer C and these segments can be related with the last four months in his rehabilitation program. As it was expected, these data proved without

**Table 3.** Concentrations of COC and BE (ng/mg) in hair segments from real drug users.

| Subject | June 2013 | | | | | | | | | | | | July 2013 | | August 2013 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I (0-1.5 cm) "proximal" | | II (1.5-3 cm) | | II (3-4.5 cm) | | IV (4.5-5 cm) | | V (5-6.5 cm) | | VI (6.5-8 cm) | | (0-1.5 cm) "proximal" | | (0-1.5 cm) "proximal" | |
| | COC | BZE | COC | BZE | COC | BZE | COC | BZE | COC | BZE | COC | BZE | COC | BZE | COC | BZE |
| *A* | 1.73 | n.q. | 2.50 | 0.50 | 2.94 | 1.06 | – | – | – | – | – | – | 1.06 | n.d. | – | – |
| *B* | 3.48 | 2.52 | 5.10 | 3.88 | 3.47 | 3.45 | 1.29 | 2.65 | 1.51 | 3.13 | 1.96 | 3.96 | – | – | – | – |
| *C* | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | – | – | – | – | n.d. | n.d. | – | – |
| *D* | 1.33 | 0.50 | 4.30 | 2.63 | – | – | – | – | – | – | – | – | 0.92 | 0.75 | 0.77 | n.q. |

n.d.: not detected.
n.q.: not quantified.

doubt this individual's abstinence and his compliance to the therapy since this volunteer ended the COC intake 10 months prior the first sampling.

As can be seen from Table 3, for volunteers A and D, COC and BZE concentrations in hair decreased from the distal to the proximal segment of the strands, and this only could be explained by the fact that incorporation from the blood supply has been stopped because the individuals abstained from consumption of COC. Figure 2 shows the electropherograms obtained for the three hair segments of the volunteer A obtained in June 2013. Even that this individual has stopped the COC consumption 3 months prior the first sampling period in our study, we still can find COC and BZE in the analyzed hair segments, and this can be attributed to a delayed incorporation from tissue deposits and/or sweat. In contrast, for the volunteer B, the COC and BZE concentrations showed irregular patterns. According to studies by Shen *et al.* [7], these results can be explained by degradation of the drugs present in hair over time because of cosmetic treatments and/or environmental agents (perm treatments, hair dye, ultraviolet light or heat). In addition, drug consumption habits of the individual may have played an important role in the differences of data obtained. There is also the possibility of conversion of COC to its metabolite as an artifact during samples preparation [4, 22]. Nevertheless, according to studies focused on this matter, COC is converted to BZE under drastic acidic or alkaline conditions and/or the extraction temperature is higher than 65°C [33]. In our work, neither of these settings was used for hair pretreatment, so the hydrolysis of COC is not probable under the used experimental conditions in our work.

The second sampling time was in July 2013 and hair samples were taken also proximal to the scalp to have information of probable drug consumption. Unfortunately, in this occasion volunteer B has denied to collaborate. In the case of the volunteer A, COC was detected without its metabolite BZE, suggesting the possibility of incorporation of COC during hair growth via diffusion from sweat, where the parent drug predominates over the metabolites, and/or from environmental factors such as the smoke and/or the powder [6, 34–35]. In the case of the volunteer D, the drugs concentrations had decreased with respect to the previous month, as reflected in Table 3. The hair sample of the volunteer C was reported as negative of both drugs.

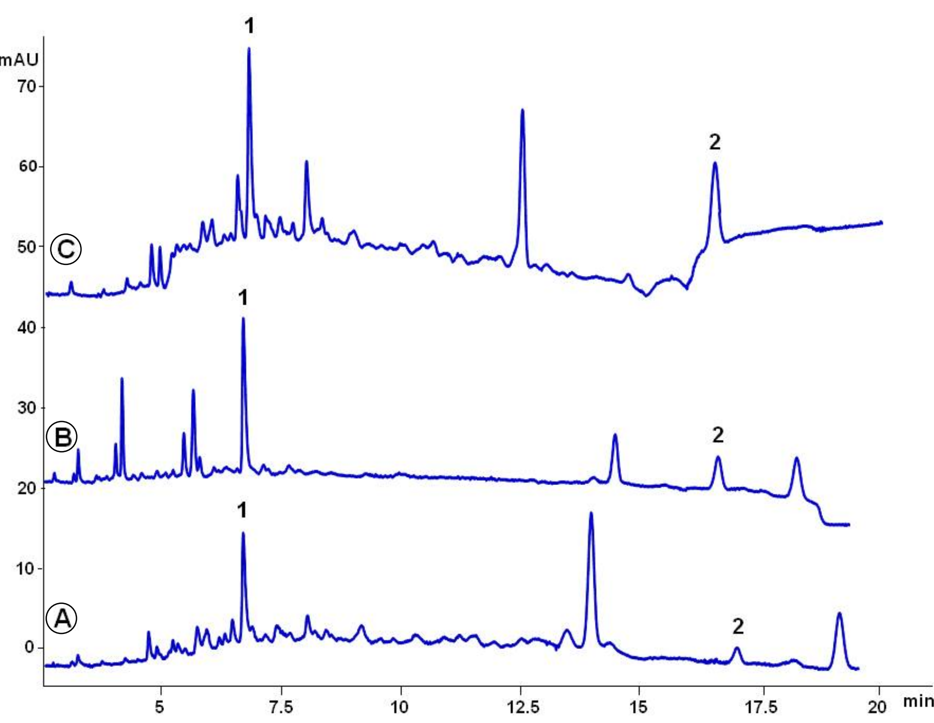


**Figure 2.** Electropherograms of the three segments of a 5 cm hair strand from subject A obtained under the optimal in-line SPE–CE-UV conditions. (A) Segment I, proximal (0-1.5 cm), (B) Segment II (1.5-3 cm) and (C) Segment III (3-4.5 cm). Peak assignments: 1, COC and 2, BZE.

These data obtained suggest that these patients abstained from consumption of COC and this only means compliance to the detoxification therapy. Finally, the last sampling period was in August 2013 and in this case, samples were also taken proximal to the scalp. In this occasion, volunteers A and C were not anymore in the center, so we could not take hair samples from them. In the case of the volunteer D, the drugs concentrations in the proximal segment sampled in July 2013 had decreased with respect to the previous month and, in August 2013, had decreased with respect to July 2013, and this fact reveals that the results obtained agree with the attempted detoxification. The concentrations found in all cases studied in this work are within the range of those published in the literature [10, 17, 31, 33, 36]. For instance, Lendoiro *et al.* [36] measured drug concentrations in the hair of 13 COC users and reported COC and BZE concentrations

(quantified by LC–MS/MS) in 50 mg of hair in the range of 0.088–20 ng/mg and 0.085–20 ng/mg, respectively. The hair samples were incubated overnight in acetonitrile to isolate the drugs followed by two cleaning steps by off-line LLE and SPE (Strata-X). Cordero *et al.* [31] analyzed hair samples from four poly-drug users enrolled on a drug rehabilitation program. The range of COC and BZE concentrations (quantified by GC–MS) found in their hair was of 1.3–5.4 ng/mg and 1.8–5.4 ng/mg, respectively. In this case, preparation of hair samples (30 mg) was performed by incubating them in 0.1 M HCl overnight, followed by an off-line SPE (mixed-mode) procedure. Another published work showed COC and BZE concentrations (quantified by GC–MS) in hair samples from 40 COC users in the range of 0.539–51.779 ng/mg and 0.035–22.073 ng/mg, respectively. The target compounds were isolated from 50 mg of hair sample by enzymatic digestion overnight followed by off-line SPE (Oasis HLB) [17]. Compared with other hair analysis methods published in the literature [11, 29–31, 36–38] the method proposed in this paper proved to be simpler, more cost-effective and highly environmentally friendly, despite that in-line SPE–CE devices are not yet commercial, which may be considered as drawbacks of this approach.

## Conclusion

A novel in-line SPE-CE method was validated with good linearity, limits of detection, repeatability, reproducibility being observed. Thereafter, this method was applied successfully to the analysis of different hair samples. This novel strategy is an attractive alternative to further exploit in the determination of drugs of abuse from different complex biological samples.

## Future perspective

Over the last few years, the utility of hair analysis for drugs of abuse has been well-established, primarily due to the several advantages that hair presents over blood and urine. Nevertheless, future research needs to be done to understand the process of incorporation of the drugs into the hair matrix. The feasibility of false positive results arising from external contamination of hair also needs to be addressed. New, more efficient and greener strategies for hair decontamination prior to analysis should be investigated further. Regarding the in-line SPE procedure, it proved to be very useful for improving the sensitivity of CE by

allowing the injection of large volumes of sample by applying the maximal injection pressure of the instrument, in other words, 930 mbar. In order to obtain larger injection volumes, the application of a higher (external) pressure could be evaluated

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### *3.1.2. Findings in the hair of drug abusers using pressurized liquid extraction and solid-phase extraction coupled in-line with capillary electrophoresis*

# FINDINGS IN THE HAIR OF DRUG ABUSERS USING PRESSURIZED LIQUID EXTRACTION AND SOLID-PHASE EXTRACTION COUPLED IN-LINE WITH CAPILLARY ELECTROPHORESIS

**Tatiana Baciu, Francesc Borrull, Carme Aguilar* and Marta Calull**

Department of Analytical Chemistry and Organic Chemistry, Rovira i Virgili University, Campus Sescelades, Marcel·lí Domingo, 1, 43007 Tarragona, Spain

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## Abstract

A suitable method has been developed and validated for simultaneously determining cocaine and its major metabolite, benzoylecgonine, 6-acetylmorphine, codeine, morphine and the methadone enantiomers in human hair samples by the in-line coupling between SPE and cyclodextrin (CyD)-assisted CE with a previous sample pretreatment procedure based on pressurized liquid extraction. Optimal separation was achieved on a fused silica-capillary of 50 µm i.d. and 80 cm total length using 11 mM-CyD in an aqueous solution of 80 mM monosodium phosphate at pH 2.5 as the separation medium and an applied voltage of 30 kV. The SPE-CE device consisted of a short length of capillary packed with Oasis HLB sorbent, which was inserted near to the inlet end of the CE capillary. Several parameters affecting the in-line preconcentration were evaluated. The LOQs reached for hair samples were in the range of 0.3–2.5 ng/mg with satisfactory analytical precision in both intraday and day-to-day experiments (RSDs <13%). Relative recoveries greater than 80% were obtained. The method has successfully been applied to the determination of these drugs of abuse in segmented hair from drug abusers who were undergoing methadone maintenance treatment. The results were consistent with the patients' statements, indicating that the method established herein can be used for verifying a history of drug abuse.

***Keywords***: *chiral CE/cyclodextrin/segmental hair analysis/drugs of abuse/pressurized liquid extraction/in-line SPE*

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## 1. Introduction

Opiates and cocaine (COC) are among the most widely abused drugs due to their ability to give the user a feeling of euphoria. As such, they represent a major social and health problem worldwide [1]. In this context, one of the most important measures to help those who become trapped in the web of addiction is the continuous establishment of detoxification treatment centres, as well as the development of new analytical methods able to monitor prescribed and non-prescribed drug use efficiently and, conversely, to verify drug abstinence during the drug treatment programmes for the evaluation of compliance and success of the treatment.

Compared to more traditional biological matrices, such as blood and urine, hair offers particular advantages, which have already been discussed elsewhere [2, 3]. Generally, scalp hair grows at an average rate of 1 cm per month and this means that each centimeter of hair records about 1 month of the individual's use or exposure to a drug [4]. Therefore, 1 cm length segmental hair analysis can provide a map of the drug consumption over time, being very useful in detoxification treatment centres. With this in mind, the purpose of this paper was to establish an analytical method for determining several illicit drugs, particularly 6-acetylmorphine (6-AM), codeine (COD), methadone (MTD), morphine (MOR), and COC and its major metabolite, benzoylecgonine (BZE), in the hair of subjects undergoing MTD maintenance treatment.

Among the analytical techniques currently used, such as GC and LC, CE has also successfully been applied to a broad range of drugs, both therapeutic and illicit, and their metabolites in a wide range of biological matrices, including human hair [3, 5]. However, as is well known, one of the most commonly cited limitations of CE is its inherent poor sensitivity. To overcome this issue, different strategies have been developed including in-line coupling between SPE and CE, which has proven to be highly useful for improving the sensitivity of CE in the analysis of different kinds of compounds, including drugs of abuse [5–7].

Hair consists of a complex structure in which the incorporation of xenobiotics is affected by its melanin content and by the lipophilic/basic nature of the drug. Within this context, various strategies have been proposed for isolating different drug groups from the hair matrix [3, 8].

To extract basic drugs, such as 6-AM, BZE, COC, COD, MOR and MTD, from hair, procedures based on dissolving by incubating/digesting the hair matrix with various extraction solvents have been widely used, such as methanol (MeOH), diluted hydrochloric acid (HCl), MeOH-acid mixtures and aqueous phosphate buffer solutions. Enzymes have also been proposed as reagents for the isolation of basic drugs from hair [3, 8]. However, recently, attention has focused on assisted extraction techniques, such as pressurized liquid extraction (PLE), to address some of the shortcomings of the extraction methods mentioned above. Within this framework, the extraction of several drugs of abuse belonging to different chemical classes from the hair matrix has successfully been performed by PLE, with extraction recoveries between 85% and 100% [9, 10].

MTD possess a stereogenic centre allowing the existence of two enantiomers (*R* and *S*) of which, as is well demonstrated today, the opioid activity is attributed to the *R*-enantiomer. In the MTD maintenance therapy of heroin addicts, both racemic MTD and *R*-MTD are applied [11]. Therefore, separate determination of each enantiomer in hair would be useful in the monitoring of patients, particularly those patients taking therapeutically the *R*-MTD and detecting both isomers in their hair samples will indicate an additional abuse of the drug.

Nowadays, CE using cyclodextrins (CyDs) as chiral selectors represents an attractive technique for resolution of enantiomers and numerous applications have been found in the analysis of different chiral drugs of abuse, including MTD, in biological samples (mainly urine) [12]. However, to the extent of our knowledge, only one paper describes the enantio-selective detection and quantification of MTD in hair by CE [13].

In view of these facts, the present work aimed to develop a cheap, simple and environmentally friendly method suitable for the simultaneous determination of 6-AM, BZE, COC, COD, MOR and the enantiomers of MTD in human hair through the in-line coupling between SPE and CyD-assisted CE with a prior sample pretreatment procedure based on PLE. The validated method was used to determine the concentrations of these drugs in hair segments from four patients undergoing a racemic MTD treatment programme with the purpose of monitoring the possible illicit use of drugs over the course of four months.

## 2. Experimental

### *2.1. Reagents and standards*

All reagents used were of analytical reagent grade. Ultrapure reagent water obtained from a water purification system (Milli-pore, Bedford, MA, USA) was used throughout this work. 6-AM, BZE, COC, COD, MOR, (*R*,*S*)-MTD and *R*-MTD were purchased from LGC Standards S.L.U. (Barcelona, Spain) as hydrochloride salts. Dichloromethane and MeOH were HPLC grade and were acquired from J.T. Baker (Deventer, the Netherlands). Ammonium hydroxide 28%, formic acid 98%, phosphoric acid 85%, HCl 37%, sodium phosphate, sodium hydroxide (NaOH), α-CyD and β-CyD were obtained from Sigma-Aldrich (MO, USA). Oasis HLB with an average particle size of 60 μm was purchased from Waters (Milford, MA, USA).

Stock standard solutions of 1,000 g/mL for each compound were prepared in MeOH and stored at −18ºC. From these standard solutions, working standard solutions of the mixture of all of the compounds at a concentration of 100 g/mL were prepared weekly by diluting in MeOH and stored at 4ºC. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard stock solution in ultrapure water.

### *2.2. Instrumentation*

The electrophoretic system was an HP$^{3D}$ CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with DAD. The analytes were detected at 200 nm. Bare fused-silica capillary with an i.d. of 50 μm and 150 μm were purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillary chamber was set at 25ºC for all of the experiments. For pH measurements, a Lab pH-meter Basic 20+ (Crison, Barcelona, Spain) was used. PLE was carried out with an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA).

### *2.3. Hair samples*

Hair samples were provided by the CAS Tarragona Drug Addiction Monitoring and Support Centre (GIPPS Health) in Tarragona, Spain. The samples were collected from four subjects (**A**, **B**, **C** and **D**) with a history of drug abuse and, at the time of sampling, they were undergoing a MTD maintenance treatment in the aforementioned centre. Adult scalp hair grows at an average rate of 1 cm per month and this means that each

centimetre of hair keeps a record of about one month of drug usage, so the specimens were collected during four consecutive months, starting the sampling in March 2015. The hair collection procedure was performed as described in a recently published review [3]. Briefly, for each subject, a strand of hair was taken and, once this was fixed with a piece of string, the hair was cut as close as possible to the scalp from the posterior vertex of the head. The samples were then rolled into aluminium foil with the root end marked and subsequently stored in paper envelopes at room temperature in a dry place until analysis.

*2.4. Sample preparation*

To prepare a hair sample for analysis, a washing procedure was applied to eliminate any possible external contamination, followed by the extraction of the analytes of interest from the hair matrix by PLE. For the sectional analysis, the strands of hair obtained were divided into several segments about 1.5 cm long from the site of cutting to the end of the strand. These were individually decontaminated twice by shaking them in 2 mL dichloromethane for 5 min each time by a vortex. After being washed, the hair segments were dried under a gentle flow of nitrogen at room temperature, weighed (100 mg each segment) and subsequently cut with scissors into segments of 1–2 mm. To prepare blank and calibration samples, pooled hair was used, prepared by mixing hair collected from several non-addicted female and male volunteers.

*2.4.1. PLE procedure*

100 mg of pooled blank hair, cut into 1–2 mm segments, was thoroughly homogenized with 1 g inert diatomaceous earth with a mortar and pestle. The mixture was then placed in an 11 mL stainless steel cell sealed at both ends with cellulose filters. The extraction was carried out with a single extraction cycle using water adjusted to pH 2.0 with HCl 37% (0.1% v/v) as the extraction solvent at 80ºC and 1,500 psi for 5 min. The preheating time was 1 min, flush volume was 0% of the cell volume and purge time was 1 min. The acidic PLE extract (12–13 mL) was transferred to a 25 mL volumetric flask, 50 μL of 28% ammonium hydroxide was then added and, finally, it was brought up to volume with Milli-Q water (final pH 9.1). Lastly, 2 mL of this solution was filtered through a 0.45 μm nylon filter, which was directly collected in a microvial for the in-line SPE-CE analysis described below (Section 2.7).

### *2.5. CE separation without preconcentration*

The CE separation was performed on a fused-silica capillary measuring 80 cm in total length (71.5 cm effective length) with an i.d. of 50 µm and an o.d. of 360 µm. The separation voltage was 30 kV (positive polarity). The background electrolyte (BGE) consisted of an aqueous solution of 80 mM monosodium phosphate and 30 mM of phosphoric acid at pH 2.5 containing 11 mM α-CyD. Prior to the first use, the capillary was conditioned with 1M NaOH for 40 min and Milli-Q water for 10 min. At the beginning of each day, the capillary was washed with 0.1M NaOH for 5 min and Milli-Q water for 5 min. The capillary was then conditioned by flushing with BGE for 10 min. Between experiments, the capillary was conditioned with 0.1 M NaOH for 4 min, Milli-Q water for 4 min and running buffer for 4 min. Standard samples were injected using the hydrodynamic mode applying a pressure of 50 mbar for 10 s.

### *2.6. Construction of the analyte concentrator*

The first step in the construction of the in-line SPE-CE device consisted of cutting 2 mm of bare fused-silica capillary of 150 µm i.d. and 360 µm o.d. A proper cut on both sides of the capillary is essential to obtain an optimum performance of the concentrator. This small piece of capillary named analyte concentrator (AC), was then introduced 1 mm into a 5 mm piece of a polytetrafluoro-ethylene (PTFE) tubing (Grupo Taper S.A., Madrid, Spain) with an i.d. of 250 µm. PTFE materials can expand hence easy to fit the o.d. of the bare fused-silica capillary. Next, a 7.5 cm segment of bare fused-silica capillary (50 µm i.d. × 360 µm o.d.) was introduced at the other end of the PTFE tubing until connecting with the AC and the free end of this capillary of 7.5 cm was connected to a vacuum pump using a syringe. Afterwards, the AC was introduced into a vial containing the Oasis HLB sorbent with an average particle size of 60 µm and this was loaded into the AC. To guarantee that the particles size of the sorbent is greater that the i.d. of the separation capillary this had previously been sieved through a 50 µm steel sieve. Later, the capillary of 7.5 cm and the AC were moved until the concentrator was placed in the half-way of the PTFE tubing. Finally, the CE separation capillary of 71.5 cm (50 µm i.d. × 360 µm o.d.) was introduced into the other part of the PTFE tubing until to join the other side of the AC. The entire process of

fabricating the concentrator was monitored under a microscope.

### *2.7. In-line SPE-CE procedure*

Before injection, the capillary with the analyte concentrator was conditioned at 930 mbar with MeOH for 5 min and Milli-Q water (adjusted to pH 9.1 with 28% ammonium hydroxide) for 5 min. The different stages of the in-line SPE procedure proposed in this paper for CE analysis of the studied drugs are described below. The first step involves the injection of standard solutions or hair extract (adjusted to pH 9.1 with 28% ammonium hydroxide) using a pressure of 930 mbar for 30 min. A sample clean-up is then performed with BGE solution by applying 930 mbar for 2 min. This step allows the removal of untrapped molecules and ensures adequate starting conditions for the separation. Afterwards, the retained analytes are eluted by injecting a plug of MeOH with 2% (v/v) of formic acid at 50 mbar for 25 s. The elution plug is then displaced from the analyte concentrator with the running buffer at 50 mbar for 250 s. Finally, a voltage of 30 kV is applied for the CE separation of the analytes.

## 3. Results and discussion

### *3.1. Optimization of the CZE separation*

According to the SciFinder database, COC and (*R*,*S*)-MTD have a pKa of 8.97 and 9.05, respectively, while, for 6-AM, the pKa values are 8.03 and 9.41, for BZE, the pKa values are 3.35 and 10.83, for COD the pKa values are 8.23 and 13.40, and finally, in the case of MOR, the pKa values are 8.25 and 9.48 [14].

Therefore, CZE separation is possible at pH values below 3.35 since, at this pH, the analytes are positively ionized so they can migrate towards the cathode.

However, previous authors have reported that opiate alkaloids and their derivatives (6-AM, COD, heroin, MOR, narcotine, noscapine, papaverine, etc.) have very similar size-to-charge ratios under acidic conditions and are difficult to separate by CZE [15–18]. To solve this issue, the addition of a CyD such as α-CyD and β-CyD to the BGE has proven to be highly useful for improving the electrophoretic separation of certain alkaloids [15–18].

Moreover, one of the goals of our study was to develop a CE-based method that allows the simultaneous determination of 6-AM, BZE, COC,

COD, MOR and the enantiomers of MTD. As is well known, enantio-selective CE separations are generally achieved by dissolving a CyD in the CE buffer.

Taking these considerations into account, the effect of adding α-CyD and β-CyD to the BGE was investigated in order to determine the most appropriate for our purpose. To do so, based on the research works cited above, α-CyD and β-CyD at an initial concentration of 6 mM were individually prepared in 80 mM monosodium phosphate at pH 2.5 and were then evaluated. The study was carried out by loading standard solutions containing the analytes at a concentration of 30 g/mL at 50 mbar for 10 s and the applied voltage was 30 kV. Figure 1A shows the electropherograms obtained.

From this figure, it can be observed that the best results in terms of resolution were obtained when α-CyD was added to the running buffer, although complete separation of *S*-MTD and *R*-MTD (peaks 5 and 6, respectively) were not accomplished. In view of these preliminary results, α-CyD was chosen to be added to the BGE for further studies.

Then, the concentration of α-CyD was examined between 6 and 12 mM in order to check if an improvement in the separation of the MTD enantiomers could be achieved. The results indicated that the chiral separation of the racemic MTD increased when the α-CyD concentration was raised to 11 mM, whereas, over this value, a decrease in the resolution achieved thus far was observed. This is explained because the separation can be affected by the decrease in the inclusion constant, which depends on the concentration of CyD. Therefore, the concentration of α-CyD selected was 11 mM.

The study of the separation voltage indicates that the application of a voltage below 30 kV led to higher migration times with no improvement in the resolution, so the separation voltage was kept at 30 kV for further experiments.

Figure 1B shows a standard electropherogram obtained under the optimal CE conditions.

### *3.2. Preconcentration by in-line SPE-CE*

Oasis HLB was selected as the SPE sorbent to enrich the studied analytes due the good results obtained for COC and its major metabolite, BZE, in our previous work focused on the analysis of hair [6]. As is well known, Oasis HLB is a polymeric sorbent with a polar group, with both hydrophilic and lipophilic retention characteristics, and it is therefore able to extract acidic, neutral and basic

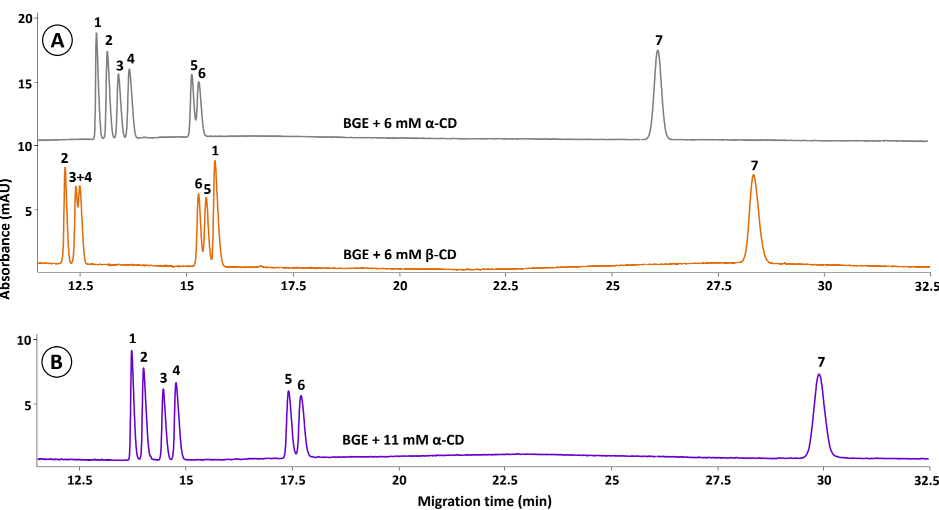


**Figure 1.** (A) Effect of the type of CyD added to the BGE on the separation of a standard solution containing the target compounds at a concentration of 30 g/mL. (B) Electropherogram obtained under optimal CE separation conditions of a standard solution containing the target compounds at a concentration of 30 g/mL. Experimental conditions are reported in the text. Peak assignments: 1) COC; 2) MOR; 3) COD; 4) 6-AM; 5) S-MTD; 6) R-MTD and 7) BZE.

compounds over a wide pH range. Based on our previous research, in which it was demonstrated that basic analytes are better retained in this sorbent at high pH [6], as well as considering the pKa values of the investigated drugs, the pH of the sample was alkalinized to 9.1 with 28% ammonium hydroxide to ensure the best conditions for retaining the analytes, since, at this pH, these are uncharged (COC and (*R*,*S*)-MTD) or zwitterionic (6-AM, BZE, COD and MOR) and their retention in the Oasis HLB is favoured MeOH containing 2% (v/v) of formic acid was chosen as the elution solvent, since, under these conditions, all of the studied drugs become positively charged and therefore poorly retained and more easily released from the Oasis HLB. This choice was also made based on our previous paper, in which the potential of acidified MeOH to desorb drugs of abuse retained in the Oasis HLB was successfully demonstrated [6].

Then, in order to achieve high sensitivity in terms of peak area, the elution volume was studied by introducing MeOH containing 2% (v/v) of formic acid for different periods of time from 5 to 30 s at 50 mbar. The results indicated that the peak area for the different compounds increased with the

increment of the elution plug time up to 25 s, whereas, beyond this point, the analytical responses in terms of the peak area remained relatively constant, probably because a plug of 25 s is enough to desorb all of the target compounds completely. Thus, 25 s was selected as the elution time for further studies, which corresponds to about 24 nL (calculated using the Poiseuille equation) of organic solvent needed for eluting all of the analytes. This makes the proposed method an attractive and environmentally sustainable analytical tool.

Injecting large volumes of sample is the simplest way to increase sensitivity and therefore obtain lower LODs. The sample loading time was evaluated between 10 and 40 min, injecting a 0.5 ng/mL mixture solution of the analytes at 930 mbar. As can be observed in Figure 2, in general, the peak areas increased with the sample loading time up to 40 min and this means that the breakthrough volume of the SPE sorbent was not exceeded. However, in the case of MOR, for injection times higher than 30 min, the analytical response in terms of the peak area remained relatively constant. As such, injection times higher than 40 min were not tested. In the end, to avoid prolonging the overall analysis time excessively, it was decided that a sample loading time of 30 min was reasonable and, therefore, this time was selected to validate the analytical method.

*3.3. Hair treatment by PLE*

Recently, Sergi *et al.* [9] have shown the potential of PLE for the extraction of several illicit drugs from hair, including COC and derivatives, amphetamines and opiates, using water-MeOH (80:20, v/v) as the extraction solvent followed by analysis by LC–MS/MS. With this in mind, one of our purposes was to develop a PLE procedure for the extraction of 6-AM, BZE, COC, COD, MOR and MTD enantiomers from hair suitable for combination with the method proposed in this paper, which involves a preconcentration step based on SPE coupled in-line with CE.

With the aim of simplifying the sample treatment procedure, our intention was to inject the hair extracts obtained after the PLE process directly into the in-line SPE-CE system for their analysis. To do so, the extraction solvent for the PLE process has to be compatible with the previously established in-line SPE procedure. However, the mixture reported by Sergi *et al.* [9] as the most suitable extracting medium in PLE for the extraction of drugs of abuse

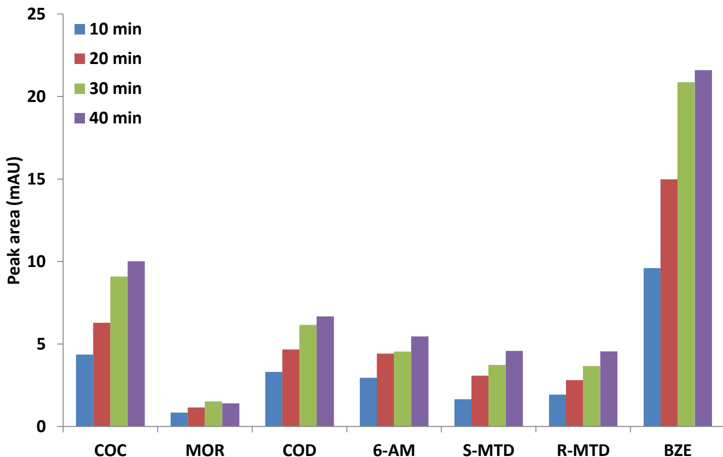


**Figure 2.** Effect of sample loading time on the peak area of the analytes by in-line SPE-CE. Experimental conditions are reported in the text. The concentration of the analytes in standard samples was 0.5 ng/mL (n = 3).

from hair is not an appropriate solvent to be used for our purpose because the presence of MeOH while the PLE extracts are loaded in the in-line SPE may lead to the undesired elution of the analytes from the SPE sorbent.

In view of this, to achieve efficient extraction of the target compounds from the hair matrix using PLE combined with in-line SPE-CE, the solvent composition was studied. Other parameters such as the temperature and static time were also researched. Pressure, flush volume and purge time could also have been investigated, but it is well known that these variables do not have a significant effect on the extraction efficiency and, therefore, they were adopted for our purposes from the work cited above. Thus, the pressure, flush volume and purge time were 1,500 psi, 0% and 1 min, respectively.

*3.3.1. Solvent*

Based on the fact that water penetrates the hair matrix, producing swelling and easy release of the drugs to the hair surface [19], pure water was investigated as the extracting medium. In addition, water adjusted to pH 2.0 was also examined in order to identify the most appropriate extraction solvent. This choice was based on a recently published study, in which a water-MeOH mixture adjusted to pH 2.0 was used as the PLE solvent, providing high extraction recoveries for several drugs of abuse, including the drugs

determined in our work, from soil and suspended particulate matter samples [20]. HCl was selected as the acidulant because it is commonly used in acidic digestions of the hair matrix to extract illicit drugs, such as 6-AM, BZE, COC, COD, MOR and MTD [3, 8].

To carry out the experiments, 100 mg of pooled blank hair, cut into 1–2 mm segments, were spiked with the drugs at a concentration of 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR. The extraction was carried out with a single extraction cycle at 40ºC for 5 min. Extracts with water (12–13 mL) were brought up to a final volume of 25 mL with Milli-Q water and subsequently adjusted to pH 9.1 with 28% ammonium hydroxide. Moreover, extracts containing water acidified with HCl 37% (0.1% v/v) were transferred to a 25 mL volumetric flask, 50 μL of 28% ammonium hydroxide was added and then brought up to volume with Milli-Q water (final pH 9.1).

Figure 3A and B shows the electropherograms of the in-line SPE-CE analysis of the hair extracts obtained using pure water and water adjusted to pH 2.0 with HCl 37% as the PLE extraction solvent, respectively. As can be seen, generally, in the case of 6-AM (peak 4) and BZE (peak 7), there was very little change in both solvents tested. In contrast, COC (peak 1) showed higher peak intensity when was extracted using acidified water, while COD (peak 3) and MOR (peak 2) provided higher peak intensities when extracted using pure water. On the other hand, *S*-MTD and *R*-MTD (peaks 5 and 6, respectively) were only recovered when acidified water was used for extraction.

In view of these preliminary results and, taking into account that, when pure water was used as the extractant, no peaks were observed for *S*-MTD and *R*-MTD, water adjusted to pH 2.0 with HCl was selected as the extraction solvent to be used in the PLE system to achieve the simultaneous determination of 6-AM, BZE, COC, COD, MOR and the enantiomers of MTD.

*3.3.2. Temperature and static time*

Four extraction temperatures between 40ºC and 100ºC were tested to find out the most appropriate for our purposes using the optimal solvent. The other PLE conditions were the same as described above. As shown in Figure 4, overall, the analytical responses in terms of the peak area for all of the tested drugs increased with the extraction temperature up to 80ºC. Above this value, in the case of BZE and MOR,

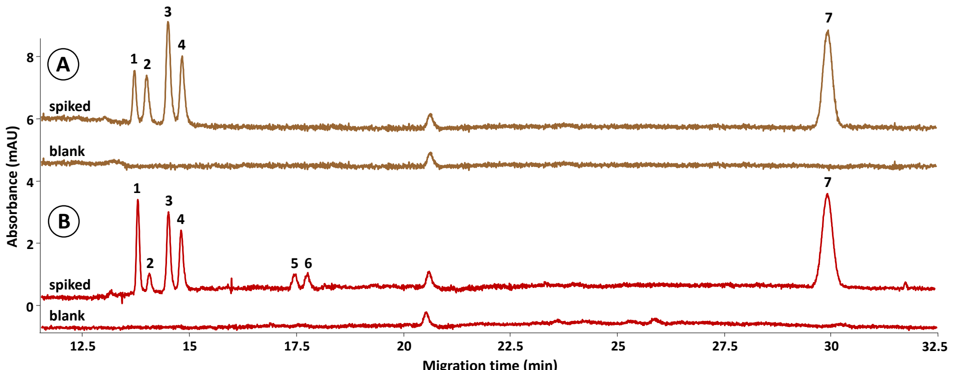


**Figure 3.** Electropherograms obtained using water (A) and water adjusted to pH 2.0 with HCl 37% (B) as the PLE extraction solvent. Other experimental conditions are reported in the text. Pooled blank hair was spiked with the examined drugs at a concentration of 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR. Peak assignments: 1) COC; 2) MOR; 3) COD; 4) 6-AM; 5) *S*-MTD; 6) *R*-MTD and 7) BZE.

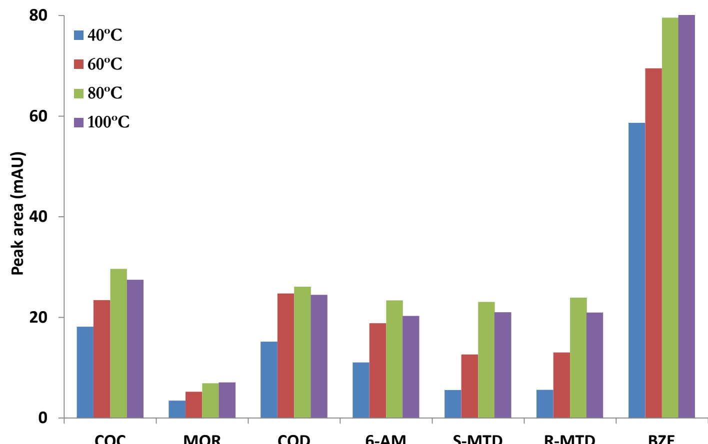


**Figure 4.** Effect of the temperature used in the PLE system on the peak area of the analytes by PLE/in-line SPE-CE. Experimental conditions are reported in the text. Pooled blank hair was spiked with the examined drugs at a concentration of 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR (n = 3).

the peak areas remained relatively constant, whereas, in the case of the others drugs, a slight decrease was observed. Within this framework, temperatures higher than 100ºC were not tested and 80ºC was selected as the optimal extraction temperature. Similarly, Baker *et al.* [20] investigated a temperature range of 40–120ºC in the extraction of several illicit drugs by PLE, including 6-AM, BZE, COC COD, MOR and MTD, from soil and suspended particulate matter samples using water-MeOH at pH 2.0 as the PLE solvent. The optimal temperature of 80ºC was selected due to the decreasing recovery of various compounds above this temperature.

Four static times between 5 and 20 min were evaluated using the optimal solvent and temperature, while the other extraction conditions were the same as mentioned above, since this parameter may affect the extraction yield. The results showed that, in our case, the static time does not have a significant impact on the extraction of the compounds from hair because, in all cases, the analytical responses regarding peak area remained relatively constant. As such, 5 min was chosen as the static time.

In view of the values chosen as optimal for extraction temperature and static time, it can be concluded that the time required to complete the pretreatment procedure developed in this paper is about 15 min (2 min for mixing with the diatomaceous earth and 13 min for PLE), which makes this methodology a reasonably attractive candidate for routine toxicological analysis in comparison with the conventional hair sample preparations mentioned in the introduction.

### *3.4. Figures of merit for hair samples*

Figure 5 shows the electropherograms obtained under the optimal PLE/in-line SPE-CE conditions of pooled blank hair (A) and pooled blank hair spiked with the studied compounds at a concentration of 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR (B). From the figure, it can be seen that no matrix peaks were found co-migrating with the analytes and clean electropherograms with little chemical background noise were obtained. This shows the specificity of the developed PLE/in-line SPE-CE method.

The proposed PLE/in-line SPE-CE method was validated in pooled blank hair samples in terms of linearity, repeatability, reproducibility, LOD, LOQ and relative recoveries, and the obtained values are presented in Table 1.

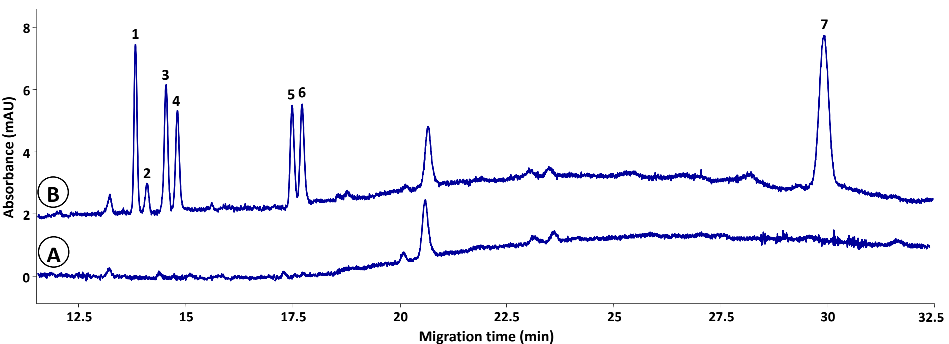


**Figure 5.** Electropherograms obtained under optimal PLE/in-line SPE-CE conditions of pooled blank hair (A) and pooled blank hair spiked with the studied compounds at a concentration of 1.25 ng/mg for 6-AM, BZE, COC, COD and (*R*,*S*)-MTD and 2.5 ng/mg for MOR (B). Experimental conditions are reported in the text. Peak assignments 1) COC; 2) MOR; 3) COD; 4) 6-AM; 5) S-MTD; 6) R-MTD and 7) BZE.

The study of linearity was carried out using pooled blank hair spiked with known amounts of each drug ranging from 0.3 to 25 ng/mg of hair, by performing three replicates of each concentration level. The calibration graphs (Table 1) with n=6 data points were constructed by plotting peak areas as a function of the concentration and were linear over the ranges shown in Table 1, with a correlation coefficient for all the analytes of >0.99.

The LODs were determined as the concentrations corresponding to three times the noise signal (n=5) and the LOQs as the lowest calibration level of each compound. The corresponding values, expressed as ng/mg of hair, are also listed in Table 1. In the case of abusers, most drugs of abuse are found in hair in the ng/mg range [3]. For example, in one comprehensive study, 34,626 samples of hair from different sources have been analysed, such as medical and legal, workplace, clinical, research, police (forensics), schools and insurance [21]. Amounts of 6-AM, BZE, COC, COD, MOR and MTD were detected in the hair specimens in the range of 0.2–63.8, 0.1–36.1, 0.2–159.9, 0.2–12.7, 0.2–41.1 and 0.8–98.2 ng/mg, respectively. In view of this and the data provided in Table 1, it can be concluded that the method developed in this work shows suitable sensitivity for determining illicit drug use in therapeutic drug monitoring, drug rehabilitation programmes, workplace and even in forensic cases.

The precision was examined at three different concentrations. Repeatability was evaluated by injecting three

replicates of each concentration level in duplicate on the same day and under identical experimental conditions. Intermediate precision was assessed over five consecutive days by injecting three replicates of each concentration level each day. The results presented in Table 1 are expressed as the %RSD of the peak areas. The recovery study was carried out by comparing the peak areas corresponding to pooled blank hair spiked with three different concentrations, with the peak areas obtained for standard solutions containing the same concentration levels, all analysed by using the developed PLE/in-line SPE-CE method. The mean extraction yields values were calculated from three independent analyses. Relative recoveries greater than 80% were obtained (Table 1). The RSD values were less than 15% (data not shown). As can be seen, the method proposed in the present work provides satisfactory results in terms of recoveries and precision.

Compared to other hair analysis methods for drug testing published in the literature [3], the method proposed in this paper is faster, simpler and more cost-effective, as well as highly environmentally friendly, although a relatively large amount of hair (100 mg) is required, which may be considered as a drawback of this approach when sample availability is limited.

### *3.5. Hair samples from drug abusers*

Sixteen hair samples from four subjects (**A, B**, **C** and **D**) undergoing an MTD maintenance treatment programme were collected over a four-month period, from March to June 2015 (at the beginning of each month), and examined for the drugs of abuse under study. All participants started their rehabilitation when they stopped using heroin. Therefore, all subjects were administered with racemic MTD throughout this study. Detailed data concerning these individuals are presented in Table 2.

Based on that scalp hair grows on average 1 cm per month, 6-AM, BZE, COC, COD, MOR, *S*-MTD and *R*-MTD concentrations were investigated in hair segments 1.5 cm long from the site of cutting (proximal segment) to the end of the strand. Each hair segment was extracted and analysed as described above. The identification of the drugs was performed taking into consideration their migration times and absorption spectra. The results obtained from the real cases are shown in Figures 6 and 7, and discussed in detail below.

**Table 1.** Regression equations, repeatability and reproducibility values, LODs and relative recoveries obtained for hair samples by PLE/in-line SPE-CE.

| | *COC* | *MOR* | *COD* | *6-AM* | *S-MTD* | *R-MTD* | *BZE* |
|---|---|---|---|---|---|---|---|
| **Linearity (ng/mg)** | 0.3−12.5 | 2.5−25.0 | 0.4−12.5 | 0.5−12.5 | 0.5−12.5 | 0.5−12.5 | 0.3−12.5 |
| **Calibration curve** [a] | y=15.531x +5.871 | y=1.180x +2.110 | y=14.252x +4.486 | y=9.920x +5.472 | y=7.483x +2.750 | y=7.463x +3.307 | y=20.524x +7.127 |
| **$r^2$** | 0.9939 | 0.9985 | 0.9969 | 0.9908 | 0.9945 | 0.9981 | 0.9981 |
| **LOD (ng/mg)** | 0.10 | 1.00 | 0.12 | 0.13 | 0.13 | 0.13 | 0.10 |
| **Repeatability (%RSD)** [b] | | | | | | | |
| 0.5 ng/mg | 9.68 | – | 9.70 | 8.62 | 7.29 | 7.84 | 9.08 |
| 2.5 ng/mg | 7.39 | 2.72 | 2.51 | 5.54 | 6.54 | 5.31 | 3.61 |
| 10.0 ng/mg | 8.33 | 3.07 | 2.48 | 5.99 | 4.60 | 4.93 | 3.03 |
| **Reproducibility (%RSD)** [c] | | | | | | | |
| 0.5 ng/mg | 11.31 | – | 8.80 | 12.78 | 10.42 | 10.46 | 8.43 |
| 2.5 ng/mg | 8.40 | 7.16 | 9.12 | 8.94 | 9.83 | 8.81 | 8.01 |
| 10.0 ng/mg | 8.70 | 8.62 | 7.08 | 7.45 | 8.52 | 9.43 | 7.16 |
| **Relative recoveries (%, *n*=3)** | | | | | | | |
| 0.5 ng/mg | 86.45 | – | 87.85 | 84.43 | 82.74 | 87.79 | 91.31 |
| 2.5 ng/mg | 89.09 | 82.93 | 88.07 | 88.12 | 90.51 | 94.56 | 89.02 |
| 10.0 ng/mg | 81.15 | 84.23 | 95.32 | 94.05 | 93.23 | 92.10 | 93.27 |

[a] y: peak area value (mAU × seconds); x: concentration (ng/mg).
[b] intra-day analysis (%, n=6).
[c] inter-day analysis (%, n=15).

**Table 2.** Characteristics of the four individuals under study.

| *Subject* | *Age* | *Sex* | *Hair treatment* | *Substance declared used* |
|---|---|---|---|---|
| **A** | 30-40 | M | No | Heroin in use for years; stopped intake about 7–8 months prior to March 2015; COC in use at the time of sampling |
| **B** | 40-50 | M | No | Heroin in use for years; stopped intake about two months prior to March 2015; COC in use at the time of sampling |
| **C** | 30-40 | F | No | Heroin in use for four years; then, from Christmas 2014, sporadic use; stopped intake about two weeks prior to March 2015 |
| **D** | 40-50 | M | No | Heroin in use for years; stopped intake about 7–8 months prior to March 2015 |

### *3.5.1. Findings in the hair of Volunteer A*

In March 2015, a strand of hair of about 1.5 cm long proximal to the scalp, which corresponds to recent drug use, was taken and then analysed. Figure 6 shows the electropherogram obtained. The results, displayed in Figure 7, indicated that the hair sample tested positive for *S*-MTD and *R*-MTD with concentrations of 4.9 and 7.2 ng/mg, respectively. This finding is consistent because racemic MTD was the prescribed drug.

Moreover, the hair sample also tested positive for MOR, with a concentration of 2.6 ng/mg. Heroin, once ingested, is primarily metabolized in the body to 6-AM, which is further hydrolysed to MOR. However, MOR may also be originated from the ingestion of therapeutic drugs such as the COD and MOR itself [22]. In this context, the Society of Hair Testing recommends differentiating heroin use from COD or MOR use by testing for the presence of 6-AM [23]. MOR was the only opiate drug detected, indicating MOR use on this occasion, although, according to interview data, the individual did not report having consumed this illicit substance.

In addition to opiates, COC and BZE with concentrations of 5.6 and 7.2 ng/mg, respectively, were also found. To prove ingestion of COC, the Society of Hair Testing recommends the detection of COC and at least one of its metabolites, mainly BZE, as well as a metabolite-to-parent drug ratio greater than 0.05 [23]. The BZE/COC ratio was of 1.3, suggesting polydrug use by this subject. This finding is in accordance with the data provided by the individual at the time of sampling.

In April, May and June 2015, the hair samples were always proximal to the scalp, which, as mentioned above, corresponds to recent drug consumption. As can be seen in Figure 7, all of the hair samples analysed were positive for BZE (2.2–11.6 ng/mg), COC (1.5–10.2 ng/mg), MOR (3.8–12.3 ng/mg), *S*-MTD (2.0–6.3 ng/mg) and *R*-MTD (3.1–8.5 ng/mg). The BZE/COC ratios ranged from 1.0 to 1.6.

The detection of COC, its metabolite BZE, and MOR proves that, in spite of abstinence requirements, the patient had been using COC and MOR throughout the whole period of this study.

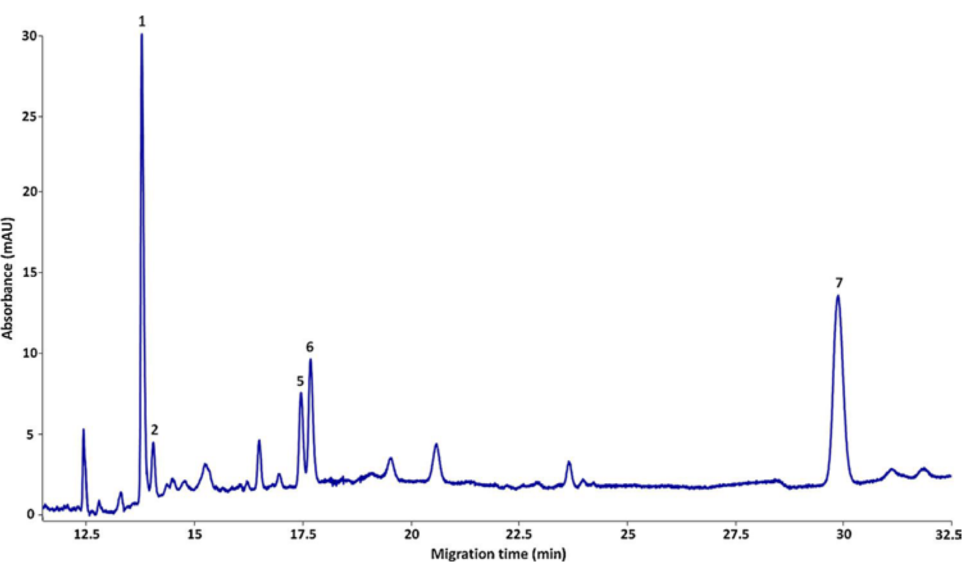


**Figure 6.** Electropherogram obtained under optimal PLE/in-line SPE-CE conditions for the hair sample of Volunteer **A** collected in March 2015. Peak assignments 1) COC; 2) MOR; 5) *S*-MTD; 6) *R*-MTD and 7) BZE.

*3.5.2. Findings in the hair of Volunteer B*

In March 2015, a strand of hair of about 1.5 cm long proximal to the scalp was taken and then analysed. The results obtained are shown in Figure 7 and indicate that the hair sample tested positive for 6-AM with a concentration of 0.8 ng/mg, which is a specific marker of heroin use. However, according to interview data, this patient had stopped consuming heroin about two months before our first sampling. Shen *et al.* [22] recently studied the disappearance of 6-AM, MOR and COD from hair after discontinuation of opiate abuse and reported that these substances could be detected in the hair of former drug users for 3–4 months after abstinence began, probably because of the delayed incorporation from tissue deposits and/or sweat.

COC and BZE with concentrations of 0.4 and 0.7 ng/mg, respectively, and, *S*-MTD and *R*-MTD with concentrations of 2.3 and 4.2 ng/mg, respectively, were also found. The BZE/COC ratio was 1.7. Subject **B** reported using COC during the MTD treatment and, therefore, our findings are reasonable and in accordance with information given by the volunteer.

When this subject was tested again in April 2015, as shown in Figure 7,

the 6-AM concentration (0.6 ng/mg) in the segment proximal to the scalp, which corresponds to recent drug use, had decreased with respect to the previous month and, in May 2015, the hair sample taken proximal to the scalp gave a negative response for this drug. The hair sample collected in June 2015 proximal to the scalp was reported as negative for 6-AM too. This can only be explained by the fact that incorporation from the blood supply had stopped, suggesting that this patient had abstained from using heroin for at least the previous four months. However, the results obtained revealed that, although enrolled in a detoxification programme, this patient has not stopped using COC. This drug and its metabolite with concentrations of 0.5–5.0 and 3.7–10.1 ng/mg, respectively, were quantified in the hair samples taken in April, May and June 2015. The BZE/COC ratios ranged from 0.7 to 11.0. *S*-MTD and *R*-MTD were found with concentrations of 3.7–4.5 and 6.8–11.1 ng/mg, respectively.

#### *3.5.3. Findings in the hair of Volunteer C*

In March 2015, a strand of hair of about 9 cm long was taken an divided into six sections of 1.5 cm in length, and then each segment of hair was individually analysed. All of the investigated hair segments tested positive for 6-AM with concentrations of 1.1–2.5 ng/mg. According to interview data, this patient joined to the rehabilitation treatment programme and had stopped consuming heroin about two weeks prior to our first sampling, which means that the segments of hair examined represent the period when the subject was consuming heroin and, therefore, our findings are consistent.

When this patient was tested again in April, May and June 2015, as illustrated in Figure 7, *S*-MTD and *R*-MTD were found in the segments proximal to the scalp with concentrations of 1.9–5.1 and 3.2–9.1 ng/mg, respectively. These results are reasonable because the hair sample collected in April 2015, represent the beginning of the treatment with racemic MTD and the hair samples collected in May and June 2015, represent the period in the rehabilitation centre.

Moreover, even though this individual had stopped consuming heroin about two weeks before our first sampling, 6-AM was still found in the proximal segments analysed in April, May and June 2015 with concentrations of 1.5, 0.9 and 0.6 ng/mg, respectively.

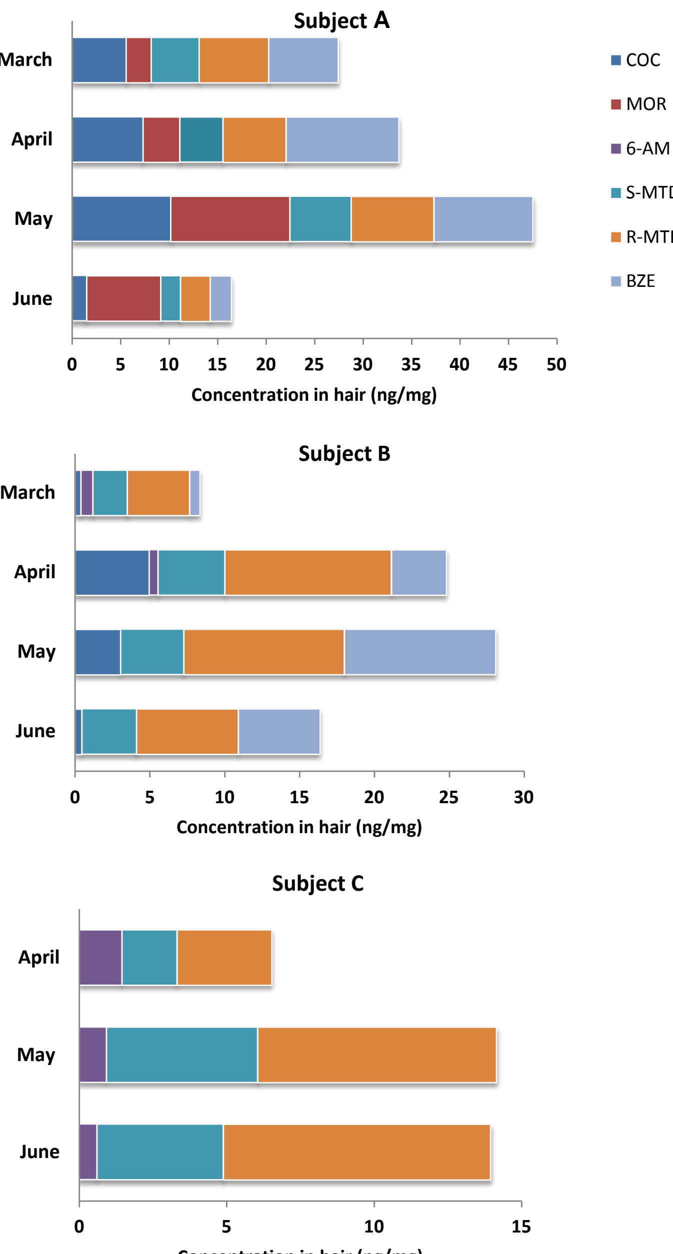


**Figure 7.** Concentrations (ng/mg) of the drugs found in the hair specimens from Volunteers **A**, **B** and **C** collected in the different months.

This evidence once again supports the results provided by Shen *et al.* [22], mentioned in Section 3.5.2. In addition, as can be observed, 6-AM concentration has gradually decreased over the three months and this can only be explained by the fact that incorporation from the blood supply had stopped because the volunteer had abstained from using heroin.

### *3.5.4. Findings in the hair of Volunteer D*

In March 2015, a strand of hair of about 8 cm long was taken and divided into five sections of 1.5 cm in length, and then each segment of hair was individually analysed. All of the investigated segments only tested positive for *S*-MTD and *R*-MTD, with concentrations of 4.7–18.5 and 7.6–20.7 ng/mg, respectively. The hair samples taken in April, May and June 2015 proximal to the scalp, which corresponds to recent drug use, also only tested positive for *S*-MTD and *R*-MTD, with concentrations of 7.9–11.3 and 13.8–20.2 ng/mg, respectively. The results obtained were in accordance with the attempted detoxification and this volunteer's compliance with the therapy. In the case of the segments of hair positive for *S*-MTD and *R*-MTD with concentrations out of the calibration range, twofold dilution of the hair extracts obtained after the PLE process was prepared and analysed with the aim to properly quantify.

From the results obtained, it can be concluded that the concentrations found in all cases studied in this work are within the range of those published in the literature. For example, Lendoiro *et al.* [24] determined opiates, COC and their metabolites, among others, in 13 hair samples from forensic cases and reported concentrations between 0.02 and 20.0 ng/mg. Cordero *et al.* [25] analysed hair samples from four polydrug users enrolled on a drug rehabilitation programme. The range of 6-AM, BZE, COC, COD and MOR concentrations found in their hair was from 1.3 to 13.0 ng/mg. Frost *et al.* [13] determined a concentration of 11 ng/mg for *R*-MTD in the specimen of one patient that was undergoing a *R*-MTD treatment. Another published work showed *R*-MTD and *S*-MTD concentrations in the hair samples of 20 patients undergoing an MTD treatment programme between 0.37 and 1.69 ng/mg and between 0.09 and 1.06 ng/mg, respectively [26].

## 4. Concluding remarks

A simple and environmentally friendly CyD-assisted CZE method

has been developed for simultaneously determining 6-AM, BZE, COC, COD, MOR and the enantiomers of MTD in human hair. The method was fully validated and successfully applied for the quantification of these drugs of abuse in segmented hair samples from drug abusers that were undergoing MTD maintenance treatment. The results obtained showed that this novel strategy, in conjunction with segmental hair analysis, provides an effective tool for determining drug abuse histories, being very useful in the monitoring of patients undergoing rehabilitation and addiction treatment programmes.

**Acknowledgement**

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UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES
Tatiana Baciu

### *3.1.3. Enantioselective determination of cathinone derivatives in human hair by capillary electrophoresis combined in-line with solid-phase extraction*

# ENANTIOSELECTIVE DETERMINATION OF CATHINONE DERIVATIVES IN HUMAN HAIR BY CAPILLARY ELECTROPHORESIS COMBINED IN-LINE WITH SOLID-PHASE EXTRACTION

**Tatiana Baciu, Francesc Borrull, Marta Calull* and Carme Aguilar**

Department of Analytical Chemistry and Organic Chemistry, Rovira i Virgili University, Campus Sescelades, Marcel·lí Domingo, 1, 43007 Tarragona, Spain

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**Abstract**

A suitable method has been developed and validated for the chiral separation and determination of *R*,*S*-mephedrone and one of its metabolites, *R*,*S*-4-methylephedrine, and *R*,*S*-methylenedioxypyrovalerone (*R*,*S*-MDPV) in human hair samples by the in-line coupling between SPE and CD-assisted CE with a previous sample pretreatment procedure based on pressurized liquid extraction. Optimum separation was achieved on a fused silica-capillary of 50 μm i.d. and 80 cm total length using 12 mg/mL β-CD in an aqueous solution of 80 mM disodium phosphate at pH 2.5 as the BGE and an applied voltage of 30 kV. The SPE-CE device consists of a short length of a capillary of 2 mm packed with Oasis HLB sorbent, which was inserted near to the inlet end of the CE capillary. Several parameters affecting the in-line preconcentration were evaluated. The LOQs reached for hair samples were 0.05 ng/mg for the enantiomers of mephedrone and its metabolite, and 0.40 ng/mg for the enantiomers of MDPV. The RSDs (%) obtained in intra- and inter-day studies were less than 10% and the relative recoveries were greater than 80%. The method established in this paper is advantageous for its simplicity, overall analysis time and ability to provide information of both enantiomers of a chiral drug in hair samples.

***Keywords:*** *chiral CE/hair analysis/in-line solid-phase extraction/pressurized liquid extraction/synthetic cathinones*

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## 1. Introduction

In recent years, synthetic cathinones, which are structurally similar to amphetamines (APs) and commonly marketed as bath salts, have become an increasingly popular and a dangerous class of designer drugs, abused by many individuals worldwide due to their easy of acquisition, variable legal status and euphoric/stimulant effects [1].

Two major ingredients of bath salts are mephedrone and methylenedioxy-pyrovalerone (MDPV) [2, 3]. Their use leads to effects similar to those of cocaine, methamphetamine and ecstasy. Adverse reactions can also occur, including increased heart rate and blood pressure, insomnia, nausea, vomiting, hallucinations, extreme paranoia and anxiety, seizures and even death [4–7]. Recently, mephedrone use has also been linked to suicide [8]. In this context, identification and quantification of such designer drugs in biological samples have gained widespread attention in toxicological analysis [9–12].

Compared to more traditional biological matrices, such as blood and urine, hair offers particular advantages, which have already been discussed elsewhere [13]. In recent years, several analytical procedures based on the use of GC and LC both with MS detection have been successfully developed for determining mephedrone and MDPV in hair samples [11, 14, 15].

Mephedrone and MDPV possess a stereogenic centre allowing the existence of two enantiomers (*R* and *S*) that can display significantly distinct pharmacokinetic and pharmacodynamic behavior implying differences in affinity to metabolizing enzymes or receptor sites [16]. Therefore, knowledge of the enantiomeric composition of these illicit substances can provide valuable assistance in interpreting drug testing results. In this context, examination of the mephedrone and MDPV enantiomers in hair can provide insights into incorporation mechanisms of these compounds into the hair matrix, which could be very helpful for a correct interpretation of findings in the hair of drug abusers. For this task, analytical methods that allow separate determination of each enantiomer in hair specimens are required. However, to the extent of our knowledge, no analytical method for chiral determination of synthetic cathinones in hair samples has been reported thus far.

Nowadays, CE represents a more versatile and less expensive technique for the resolution of enantiomers than LC using chiral column because, as is well known, enantio-selective CE

separations are achieved by simply dissolving a chiral selector such as a CD in the CE buffer. In this way, CE has found numerous applications in the analysis of different chiral analytes, including APs, in biological samples (mainly urine) [17, 18].

CE using CDs, particularly, native β-CD, its derivatives sulphated-β-CD, hydroxypropyl-β-CD and sulfobutyl-ether-β-CD, has also proven to be an effective tool for the enantio-separation of cathinones, including *R*,*S*-mephedrone and *R*,*S*-MDPV [19–23]. These reports mainly focus on the optimization of the enantio-separation of different synthetic cathinones and two of them described the qualitative chiral analysis of these substances in seized drug samples [22, 23]. In this context, to the best of our knowledge, no CE-based assay has been reported to date for the chiral detection and/or quantification of cathinone derivatives in biological samples. This fact prompted us to continue working in this field and, therefore, the purpose of this paper was to establish a chiral CE-based method for the separation and quantitative determination of cathinone drugs in a biological matrix, particularly human hair.

Freeing drugs bound within the hair matrix is commonly achieved either by acidic or alkaline digestion, solvent extraction, enzymatic hydrolysis or incubation with various buffer systems [13, 24]. However, in recent years, attention has focused on assisted extraction techniques, such as pressurized liquid extraction (PLE), to address some of the shortcomings of the classical hair sample preparations. Within this context, the extraction of several drugs of abuse from the hair matrix has been successfully performed by PLE with extraction recoveries between 85% and 100% [25, 26].

However, as is well recognized, one of the most commonly cited limitations of CE is its inherent poor sensitivity, especially when DAD is used [27, 28]. To overcome this problem, different strategies have been established, among which the in-line coupling between SPE and CE has proven to be highly useful for improving the sensitivity of CE in the analysis of recreational illicit drugs, such as cocaine and opium-related compounds, in different biological matrices [29, 30]. In addition, the use of in-line SPE in combination with CE has been demonstrated to be a highly environmentally friendly, simple and cheap approach.

In view of these facts, the present work aimed to develop an inexpensive, green and easily performed method for the chiral separation and determination of *R*,*S*-mephedrone, *R*,*S*-4-methylephedrine

(mephedrone metabolite) and *R,S*-MDPV in hair samples by the in-line coupling between SPE and CD-assisted CE with a prior sample pretreatment procedure based on PLE. Chemical structures of the studied compounds are depicted in Figure 1. In the case of mephedrone, one of its metabolites was included in this study to evaluate the behaviour of this one in the hair matrix because, in general, limited data concerning the metabolism of the synthetic cathinones exist in the scientific literature.

## 2. Materials and methods

### 2.1. Reagents and standards

All reagents used were of analytical-reagent grade. Ultrapure reagent water purified by a Milli-Q gradient system (Millipore, Bedford, MA, USA) was used throughout this work. *R,S*-mephedrone, *R,S*-4-methyl-ephedrine and *R,S*-MDPV were purchased from LGC Standards S.L.U. (Barcelona, Spain) as hydrochloride salts with a purity of 98%. Dichloromethane (DCM) and methanol (MeOH) were HPLC grade and were acquired from J.T. Baker (Deventer, the Netherlands). Ammonium hydroxide 28%, formic acid 98%, phosphoric acid 85%, hydrochloric acid 37%, disodium phosphate 99%, sodium hydroxide (NaOH) 97%, α-CD 98%, β-CD 97%, γ-CD 98% and sulphated-β-CD (12-15 mol per mol β-CD) were obtained from Sigma-Aldrich (MO, USA). Oasis HLB with an average particle size of 60 μm was purchased from Waters (Milford, MA, USA).

Stock standard solutions of 1,000 μg/mL for each compound were prepared in MeOH and stored at -18ºC. From these standard solutions, working standard solutions of the mixture of all of the compounds at a concentration of 100 μg/mL were prepared weekly by diluting in MeOH and they were stored at 4ºC. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard stock solution in ultrapure water.

### 2.2. Instrumentation

The electrophoretic system was a HP$^{3D}$ CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a DAD. The analytes were detected at 200 nm. Bare fused-silica capillary with an i.d. of 50 and 150 μm were purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillary chamber was set at 25ºC for all the experiments. For pH measurements, a Lab pH-meter Basic 20+ (Crison, Barcelona,

**Figure 1.** Chemical structures of the studied cathinone derivatives.

Spain) was used. The PLE was carried out with an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA).

### 2.3. Sample preparation

To prepare a hair sample for analysis, a washing procedure was applied to eliminate any possible external contamination followed by the extraction of the target compounds from the hair matrix by PLE. Hair (100 mg) was placed in a 10 mL glass tube and 2 mL DCM was added. The mixture was vortex mixed for 5 min and, after removal of the organic solvent, another 2 mL DCM was added and the same procedure was repeated. After being washed, the hair was dried under a gentle nitrogen flow at room temperature and subsequently cut with scissors into segments of 1-2 mm.

To prepare blank and calibration samples, pooled hair prepared by mixing hair samples collected from several non-addicted females and males volunteers was used.

#### 2.3.1. PLE procedure

100 mg of pooled blank spiked hair, cut into 1-2 mm segments, were thoroughly homogenized with 1 g inert diatomaceous earth with a mortar and pestle. The mixture was then placed in an 11 mL stainless-steel cell that was sealed at both ends with cellulose filters. The extraction was carried out with one cycle using Milli-Q water alkalinized to pH 10 with 28% ammonium hydroxide at room temperature and 1,500 psi for 5 min. The flush volume was 0% of the cell volume and the purge time was 1 min. The aqueous PLE extract (12-13 mL) was transferred to a 25 mL volumetric flask that was then brought up to its volume with Milli-Q water adjusted to pH 10 with 28% ammonium hydroxide. Finally, 2 mL of this solution was filtered through a 0.22 μm polytetrafluoroethylene filter, which was directly collected into a microvial for the in-line SPE-CE analysis described below.

### 2.4. CE separation without preconcentration

The CE separation was performed on a fused-silica capillary measuring 80 cm in total length (71.5 cm effective length) with an i.d. of 50 µm and an o.d. of 360 µm. The separation voltage was 30 kV (positive polarity). The BGE consisted of an aqueous solution of 80 mM disodium phosphate adjusted with phosphoric acid to pH 2.5, containing 12 mg/mL β-CD. Prior to the first use, the capillary was conditioned with 1 M NaOH for 40 min and Milli-Q water for 10 min. At the beginning of each day, the capillary was washed with 0.1 M NaOH for 5 min and Milli-Q water for 5 min. The capillary was then conditioned by flushing with BGE for 10 min. Between experiments, the capillary was conditioned with 0.1 M NaOH for 4 min, Milli-Q water for 4 min and running buffer for 4 min. Standard samples were injected using the hydrodynamic mode applying a pressure of 50 mbar for 5 s.

### 2.5. Construction of analyte concentrator

The first step in the construction of the in-line SPE-CE device consisted of cutting 2 mm of bare fused-silica capillary of 150 µm i.d. and 360 µm o.d. A proper cut on both sides of the capillary is fundamental to obtain an optimum performance of the concentrator. This small piece of capillary named analyte concentrator (AC), was then introduced 1 mm into a 5 mm piece of a polytetrafluoro-ethylene (PTFE) tubing (Grupo Taper S.A., Madrid, Spain) with an i.d. of 250 µm. PTFE materials can expand hence easy to fit the o.d. of the bare fused-silica capillary. Next, a 7.5 cm segment of bare fused-silica capillary (50 µm i.d.×360 µm o.d.) was introduced at the other end of the PTFE tubing until connecting with the AC and the free end of this capillary of 7.5 cm was connected to a vacuum pump using a syringe. Afterwards, the AC was introduced into a vial containing the Oasis HLB sorbent with an average particle size of 60 µm and this was loaded into the AC. To guarantee that the particles size of the sorbent are about 60 µm this had previously been sieved through a 50 µm steel sieve. Later, the capillary of 7.5 cm and the AC were moved until the concentrator was placed in the half-way of the PTFE tubing. Finally, the CE separation capillary of 71.5 cm was introduced into the other part of the PTFE tubing until to join the other side of the AC. The entire process of fabricating the concentrator was monitored under a microscope.

### 2.6. In-line SPE-CE procedure

Before injection, the capillary with the analyte concentrator was conditioned at 930 mbar with MeOH for 5 min and Milli-Q water (adjusted to pH 10 with 28% ammonium hydroxide) for 5 min. The different stages of the in-line SPE procedure proposed in this paper for CE analysis of the studied drugs are as follows. The first step involves the injection of standard solutions or hair extracts (adjusted to pH 10 with 28% ammonium hydroxide) using a pressure of 930 mbar for 40 min. A sample clean-up is then performed with BGE solution by applying 930 mbar for 2 min. This step allows the removal of untrapped molecules and ensures adequate starting conditions for the separation. Afterwards, the retained analytes are eluted by injecting a plug of MeOH with 2% (v/v) of formic acid at 50 mbar for 20 s. The elution plug is then displaced from the analyte concentrator with the running buffer at 50 mbar for 250 s. Finally, a voltage of 30 kV is applied for the CE separation of the analytes.

## 3. Results and discussion

This section describes the optimization and validation process of the proposed in-line SPE-CE method for the enantioselective determination of *R*,*S*-mephedrone, *R*,*S*-4-methylephedrine and *R*,*S*-MDPV in hair samples.

### 3.1. Chiral separation optimization

This study was carried out by loading standard solutions containing the analytes at a concentration of 50 μg/mL at 50 mbar for 5 s. Other experimental conditions were the same as those described in Section 2.4.

In the initial experiments, the achiral separation of the target compounds was first evaluated using disodium phosphate at different concentrations between 50 and 100 mM without the chiral selector as the CE separation medium. This choice was based on previously published studies in which sodium phosphate buffers were used to analyse different illicit drugs such as cocaine and its metabolites, opiates/opioids and APs by CE [31]. As the studied drugs are weak bases, with p*Ka* values between 7.4 and 9.5 [32, 33], the pH of the BGE was adjusted to 2.5 with phosphoric acid because, at this pH, the analytes are positively charged so they can migrate towards the cathode. A disodium phosphate concentration of 80 mM was selected because better resolution was obtained without an excessive increase in the Joule effect

for the achiral CE separation of the cathinone derivatives under study with a separation voltage of 30 kV and a capillary temperature of 25ºC (Figure 2A).

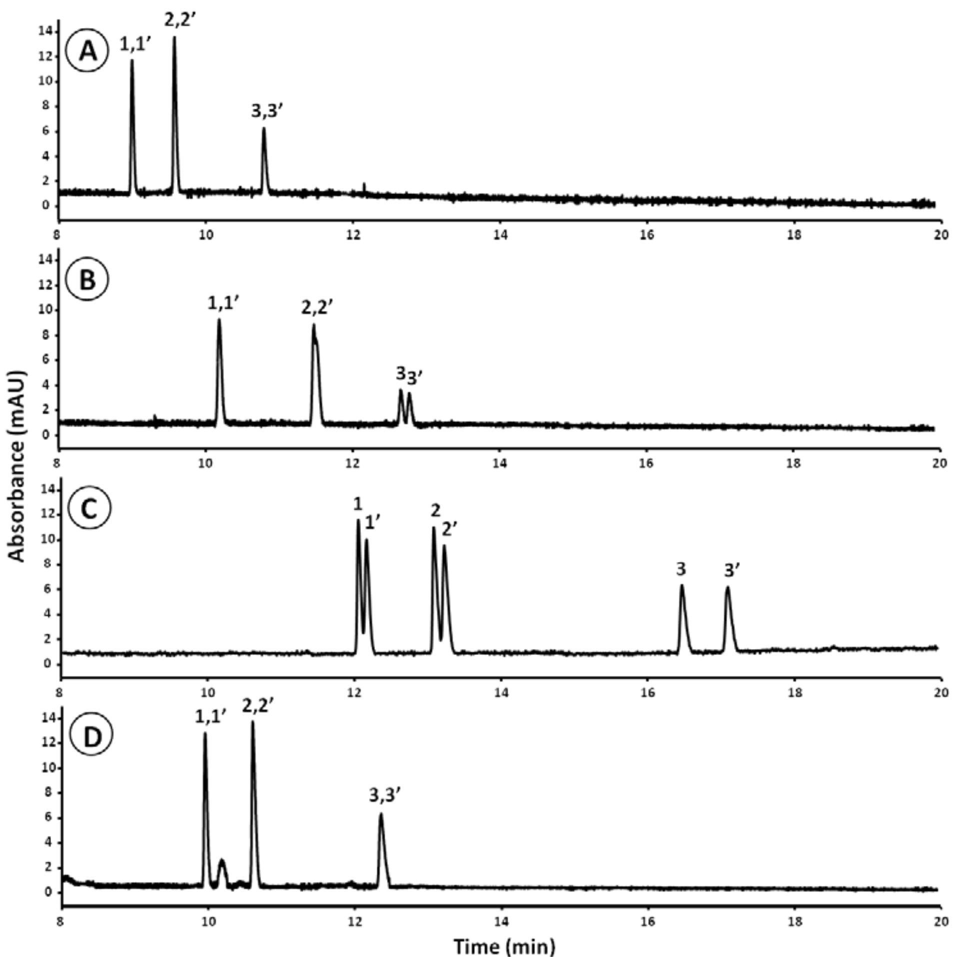


**Figure 2.** Electropherograms showing the separation of a standard solution containing the target compounds at a concentration of 50 µg/mL using (A) no chiral selector, (B) 6 mg/mL α-CD, (C) 6 mg/mL β-CD and (D) 6 mg/mL γ-CD as the chiral selector. CE conditions: 80 mM disodium phosphate solution adjusted with phosphoric acid to pH 2.5 as the BGE, hydrodynamic injection applying 50 mbar for 5 s, cassette temperature of 25ºC, separation voltage of +30 kV and a detection wavelength of 200 nm. Peak assignments: (1,1') *R,S*-mephedrone; (2, 2') *R,S*-4-methylephedrine and (3, 3') *R,S*-MDPV.

Chiral CE separations of synthetic cathinone drugs, including *R,S*-mephedrone and *R,S*-MDPV, have been recently reported in several studies using both native and modified CDs [19-23]. With this in mind, three native CDs, namely α-, β- and γ-CD, and one CD derivative, sulphated-β-CD, were investigated to find the most appropriate chiral selector for our purpose. To do so, based on the research works cited above, α-, β- and γ-CD, and sulphated-β-CD at an initial concentration of 6 mg/mL were all individually prepared in 80 mM disodium phosphate at pH 2.5.

Figures 2B, C and D show the electropherograms obtained when α-, β- and γ-CD, respectively, were used as the chiral selectors, and on the other hand, Figure 3 shows the electropherograms obtained when sulphated-β-CD was used for the same task. Because the sulphated-β-CD is negatively charged at low pH and migrates towards the anode, experiments in both normal (Figure 3A) and reversed (Figure 3B) polarity were carried out when this was added to the running buffer to investigate the effect of the polarity of the applied voltage on the separation.

In view of these preliminary results, the best chiral resolution for all of the target compounds was obtained when β-CD was used. This finding supports a previous study [23] in which the chiral separation of 12 cathinone analogs was achieved using β-CD in a phosphate buffer at pH 2.5. In addition, it should be pointed out that our experience shows that the use of the sulphated-β-CD in a low pH buffer did not work out as expected, although Mohr *et al.* [19] obtained the optimal separation of 19 cathinone derivatives using this CD in an ammonium acetate buffer at pH 4.5. Thus, β-CD was chosen as the suitable chiral selector for further studies.

Next, β-CD concentrations between 6 and 12 mg/mL were examined to improve the enantiomeric separation of *R,S*-mephedrone and *R,S*-4-methylephedrine. According to the literature, a concentration of 12 mg/mL is close to the limiting aqueous solubility of β-CD [22,34] and, therefore, higher concentrations were not investigated. The results indicated that the chiral separation of the racemic mephedrone and its racemic metabolite increased when the β-CD concentration was raised up to the maximum level evaluated, so the selected concentration of β-CD in this paper was of 12 mg/mL.

Finally, a capillary temperature higher than 25ºC and an applied voltage below 30 kV were also examined, although the best results in terms of chiral resolution and

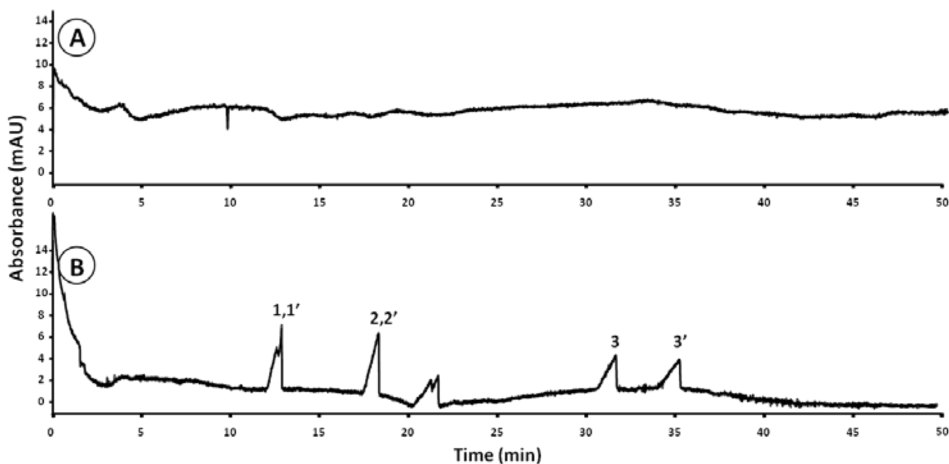


**Figure 3.** Electropherograms showing the chiral separation of a standard solution containing the target compounds at a concentration of 50 μg/mL using 6 mg/mL sulphated-β-CD as the chiral selector at (A) normal polarity of 30 kV and (B) reverse polarity of 30 kV. Other CE conditions as in Figure 1. Peak assignments (1,1') *R*,*S*-mephedrone; (2, 2') *R*,*S*-4-methylephedrine and (3, 3') *R*,*S*-MDPV.

migration times were achieved at 30 kV applied voltage and 25°C, so these values were chosen as suitable for our purpose.

As discussed in the introduction, the chirality of cathinone derivatives resulted in the need for the development of stereoselective analytical methods suitable for their efficient determination. As such, single enantiomers must be available for the correct assignment of the *R*- and *S*-forms. However, nowadays, this is particularly challenging in case of the synthetic cathinones, which, as far as we know, are sold as racemic mixtures and the pure *R*- or *S*-isomers are not commercially available for purchase. In the present paper, successful enantioselective separation of three cathinone designer drugs was achieved, but it was not possible to identify which enantiomer was the *R* and which was the *S* of the corresponding racemic pairs due to the aforementioned issue. Further work should focus on this field.

### 3.2. Preconcentration by in-line SPE-CE

Oasis HLB was selected as the SPE sorbent to enrich the compounds under study. This choice was made based on the chemical structure of the investigated analytes and the good results obtained for drugs of abuse,

such as cocaine, benzoylecgonine and some opiates/opioids, in previous works from our laboratory focused on the analysis of biological samples by in-line SPE-CE [27, 28]. Oasis HLB is a polymeric sorbent with a polar group, with both hydrophilic and lipophilic retention characteristics, and therefore able to extract acidic, neutral and basic compounds over a wide pH range. Based on our previous work, in which it was demonstrated that basic analytes are better retained in this sorbent at high pH [29], as well as considering the p*Ka* values of the examined drugs (between 7.4 and 9.5), the pH of the sample was alkalinized to 10 with 28% ammonium hydroxide to ensure the most proper conditions for retaining the analytes, since, at this pH value, these are uncharged and their retention in the Oasis HLB is favoured.

MeOH containing 2% (v/v) of formic acid was chosen as the elution solvent, since, under these conditions, all the studied drugs become positively charged and therefore, poorly retained and more easily released from the Oasis HLB. This choice was also made based on our previous paper, in which the potential of acidified MeOH to desorb drugs of abuse retained in the Oasis HLB was successfully demonstrated [29].

The elution volume was then studied by introducing MeOH containing 2% (v/v) of formic acid with a hydrodynamic injection for different periods of time from 5 to 30 s at 50 mbar to obtain high sensitivity in terms of peak area. Higher elution plugs of acidified MeOH were not tested to avoid current instability and breakdown during the CE separation. The results showed that the peak area for the different compounds increased with the increment of the elution plug time up to 20 s, whereas, over this time, the analytical responses regarding peak area remained relatively constant, probably because a plug of 20 s is enough to desorb all the target compounds completely. In the end, 20 s was selected as the elution time for further studies, which corresponds to about 20 nL (calculated using the Poiseuille equation) of organic solvent needed for eluting all of the analytes. This makes the proposed method an attractive and environmentally sustainable analytical tool.

Injecting large volumes of sample is the simplest way to increase sensitivity and therefore obtain lower LODs. The sample loading time was evaluated between 15 and 40 min, injecting a 0.5 ng/mL mixture solution of the target analytes at 930 mbar. As shown in Figure 4, in

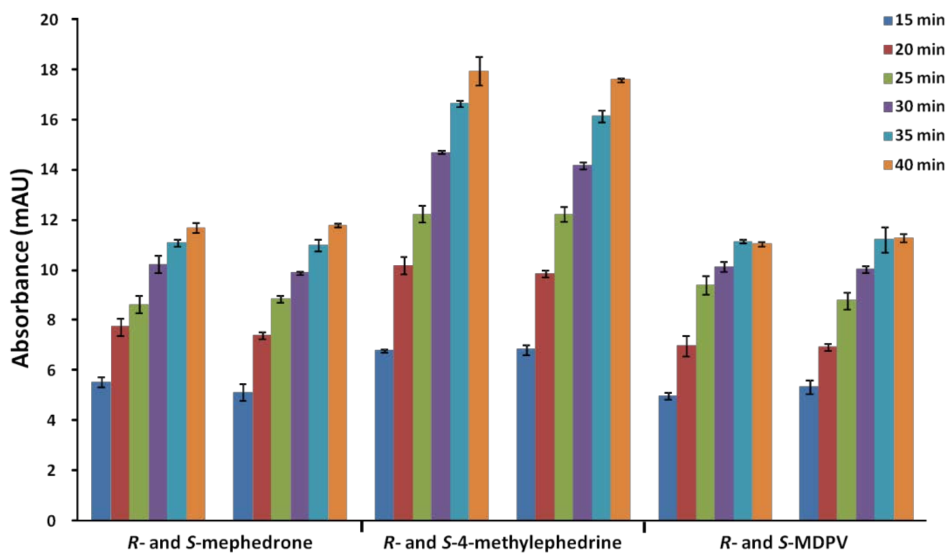


**Figure 4.** Effect of sample loading time on the peak area of the analytes by in-line SPE-CE. Experimental conditions are reported in the text. The concentration of the analytes in standard samples was 0.5 ng/mL (*n*=3).

general, the peak areas increased with the sample loading time up to 40 min, and this means that the breakthrough volume of the SPE sorbent was not exceeded. However, in the case of *R*- and *S*-MDPV, for injection times higher than 35 min, the analytical response in terms of the peak area remained constant. As such, injection times higher than 40 min were not tested. Therefore, a sample loading time of 40 min was selected to validate the analytical method.

### 3.3. Hair treatment by PLE

Recently, Sergi *et al.* [25] have successfully developed a PLE procedure for the quantitative recovery of different illicit drugs, including several APs, from hair matrix. As mentioned in the introduction, the synthetic cathinones are analogues of APs, so similar behaviour would be expected. With this in mind, based on the PLE protocol presented by Sergi *et al.* [25], attempts were made to develop a PLE method suitable for the extraction of cathinone designer drugs from hair samples.

To achieve the efficient extraction of the target compounds from the hair matrix using the PLE system, the main extraction parameters were optimized, such as the solvent,

temperature and static time. Pressure, flush volume and purge time can also be investigated, but it is well known that these parameters do not have a significant effect on the extraction efficiency and, therefore, they were adopted for our purpose from the work of Sergi *et al.* [25]. Thus, the pressure, flush volume and purge time were 1,500 psi, 0% and 1 min, respectively.

### 3.3.1. Solvent

The method proposed in this paper involves a preconcentration step based on SPE coupled in-line with CE after sample treatment by PLE. Thus, our intention was to inject the hair extracts obtained after the PLE process directly into the in-line SPE-CE system for their analysis. This played an important role when selecting the extraction solvent for the PLE, since it has to be compatible with the in-line SPE procedure developed. Sergi *et al.* [25] reported water-MeOH 80:20 (v/v) as the most suitable extracting medium in PLE for the extraction of 14 drugs of abuse, including 5 APs, from the hair matrix. However, this mixture was discarded in our study because the presence of MeOH during the loading of the PLE extracts in the in-line SPE can lead to the undesired elution of the analytes from the SPE sorbent since this organic solvent is an appropriate eluent for desorption of cathinone drugs from the Oasis HLB.

As reported in the literature, water penetrates the hair matrix, producing swelling and easy release of the drugs to the hair surface [35]. In this sense, water seems to be reasonably suitable for extracting drugs of abuse from the hair samples. In addition, water is cheap and environmentally friendly. Therefore, pure water was chosen to be tested in the first instance as the extraction solvent. Other initial conditions were as follows: one extraction step, 100ºC temperature, 1 min of preheating time and 5 min of static time.

For this study, 100 mg of pooled blank hair, cut into 1-2 mm segments were spiked with the drugs at a concentration of 0.15 ng/mg for *R*,*S*-mephedrone and its metabolite, and 0.50 ng/mg in the case of *R*,*S*-MDPV and then extracted by PLE under the conditions described above. The aqueous extract (12-13 mL) was transferred to a 25 mL volumetric flask that was brought up to its volume with Milli-Q water and then adjusted to pH 10 with 28% ammonium hydroxide.

In Figure 5A, it can be seen that, when pure water was used as the extractant, very small peaks were obtained for *R*- and *S*-MDPV,

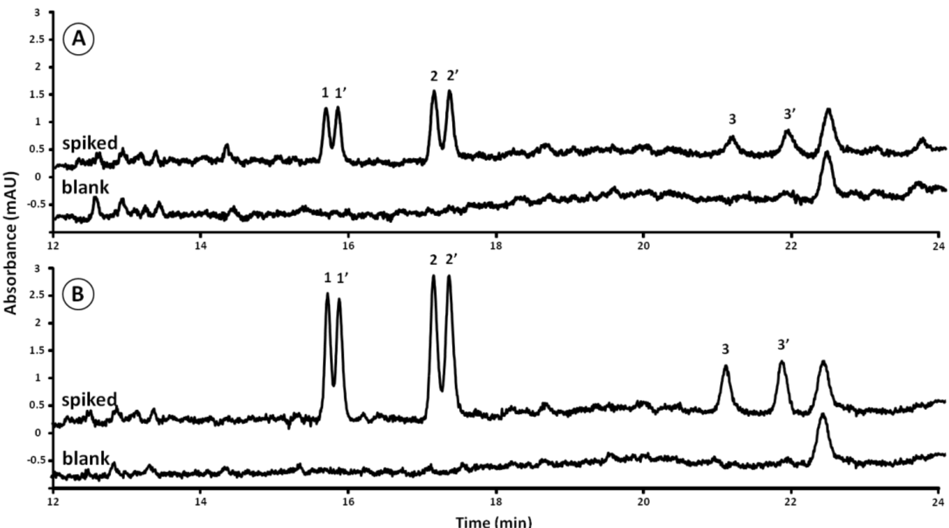


**Figure 5.** Electropherograms obtained using (A) water and (B) water adjusted to pH 10 with 28% ammonium hydroxide as the PLE extraction solvent. Other experimental conditions are reported in the text. Pooled blank hair was spiked with the examined drugs at a concentration of 0.15 ng/mg for *R*,*S*-mephedrone and its metabolite, and 0.50 ng/mg for *R*,*S*-MDPV. Peak assignments (1,1') *R*,*S*-mephedrone; (2, 2') *R*,*S*-4-methylephedrine and (3, 3') *R*,*S*-MDPV.

suggesting that, under these conditions, the two isomers were poorly released from the hair matrix. Therefore, to try to improve the extraction of the MDPV enantiomers from hair, bearing in mind that the drugs under study are basic compounds, water adjusted to a basic pH was also investigated as the extracting medium. In this context, taking into account that, in this work, the PLE extracts are directly injected into the in-line SPE-CE system and the pH of the sample is 10, as well as considering the p*Ka* values of the target analytes (between 7.4 and 9.5), Milli-Q water alkalinized to pH 10 with 28% ammonium hydroxide was examined under the same conditions stated above. The basic aqueous extract (12-13 mL) was transferred to a 25 mL volumetric flask that was then brought up to its volume with Milli-Q water adjusted to pH 10 with 28% ammonium hydroxide. The use of basified Milli-Q water as the extraction solvent, as shown in Figure 5B, proved to increase the peak intensity not only in the case of the MDPV enantiomers but also of the enantiomers of mephedrone and its metabolite. Probably, under these conditions, as the pH equilibrium is affected, an increased solubilization of

all the target compounds into the basic aqueous medium occurred. Therefore, Milli-Q water adjusted to pH 10 was chosen as the extraction solvent to be used in the PLE system.

### 3.3.2. Temperature and static time

As is widely known, the use of high temperatures in PLE may, on one hand, increase extraction yield but, on the other hand, lead to the degradation of the analytes. For that, extraction temperatures between 25ºC and 100ºC were tested to find out the most appropriate for our purpose using the optimal solvent and the same conditions described thus far. As shown in Figure 6, the peak areas for all of the tested designer drugs increased as the extraction temperature decreased. In this respect, temperatures higher than 100ºC were not tested and 25ºC was selected as the optimal extraction temperature. To the extent of our knowledge, the thermal behaviour of the synthetic cathinones during the sample preparation procedure of the hair matrix has not been thoroughly investigated so far. However, Shah *et al.* [36] reported something related to this issue, particularly that digestion of the hair with 1 mL of 1 M NaOH solution at 95ºC for 10 min led to the complete degradation of mephedrone and two of its metabolites. In view of this, probably the analytes studied herein decompose with increasing the temperature in the PLE process.

Four static times between 5 and 20 min were evaluated, since this parameter may affect the extraction yield. However, the experiments showed that, in our case, the static time does not have a significant impact on the extraction of the compounds from the hair because, in all cases, the analytical responses regarding peak area remained relatively constant. As such, 5 min was chosen as the static time.

The PLE procedure developed in the present work has several advantages over the classical hair extraction methods mentioned in the introduction. One of these is the hugely reduced time in terms of the sample preparation (about 8 min total: 2 min for mixing with the diatomaceous earth and 6 min for PLE), since the conventional procedures generally involve periods of 16-20 h [25, 26]. Another advantage is the reduced cost, mainly when compared to enzymatic hydrolysis, which is rather expensive and, in most reported cases, is not the selected procedure [25]. Finally, it is green and automated without the need for additional steps, such as the evaporation and reconstitution, mainly when compared to procedures involving the use of organic solvents.

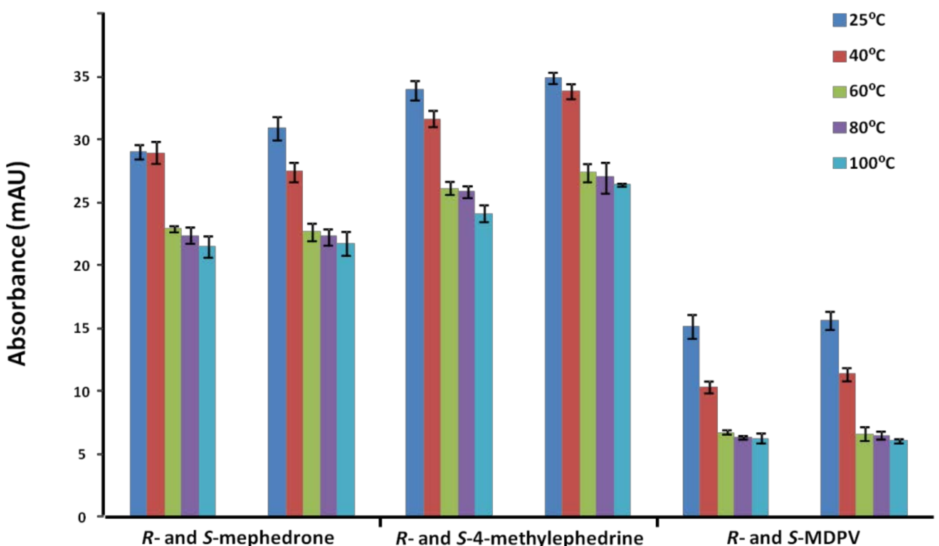


**Figure 6.** Effect of temperature used in the PLE system on the peak area of the analytes by PLE/in-line SPE-CE. Experimental conditions are reported in the text. Pooled blank hair was spiked with the examined drugs at a concentration of 0.15 ng/mg for *R*,*S*-mephedrone and its metabolite, and 0.50 ng/mg for *R*,*S*-MDPV (*n*=3).

### 3.4. Figures of merit for hair samples

Figure 7 shows the electropherograms obtained under the optimal PLE/in-line SPE-CE conditions of pooled blank hair (A) and pooled blank hair spiked with the studied compounds at a concentration of 0.15 ng/mg for *R*,*S*-mephedrone and its metabolite, and 0.50 ng/mg in the case of *R*,*S*-MDPV (B). From the figure, it can be seen that no matrix peaks were found co-migrating with the analytes, and clean electropherograms were obtained with little chemical background noise. This shows the specificity of the developed PLE/in-line SPE-CE method.

The proposed PLE/in-line SPE-CE method was validated in pooled blank hair spiked with the analytes in terms of linearity, repeatability, reproducibility, LOD, LOQ and relative recoveries, and the obtained values are shown in Table 1.

The study of linearity was carried out in pooled blank hair spiked with known amounts of each drug ranging from 0.05 to 10.0 ng/mg of hair, by performing three replicates at each concentration level. The calibration graphs, with *n*=6 data points, were

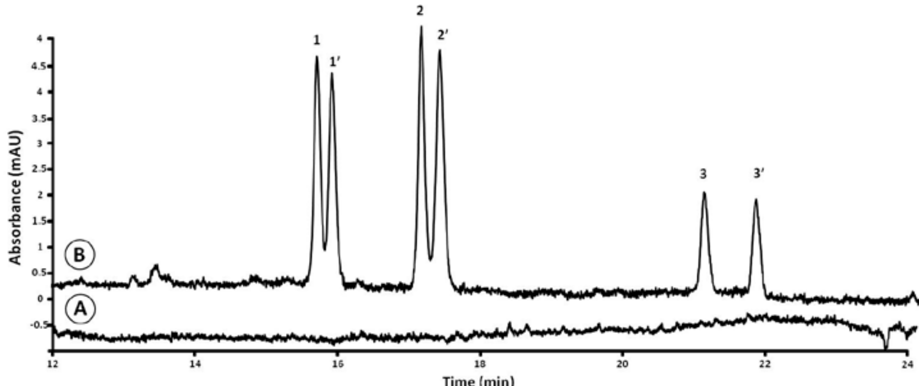


**Figure 7.** Electropherograms obtained under the optimal PLE/in-line SPE-CE conditions of pooled blank hair (A) and pooled blank hair spiked with the studied compounds at a concentration of 0.15 ng/mg for *R,S*-mephedrone and its metabolite, and 0.50 ng/mg for *R,S*-MDPV (B). Experimental conditions are reported in the text. Peak assignments (1,1') *R,S*-mephedrone; (2, 2') *R,S*-4-methylephedrine and (3, 3') *R,S*-MDPV.

constructed by plotting peak areas as a function of the concentration and are summarized in Table 1. As can be seen in this table, good linearity was obtained with regression coefficients ($r^2$) greater than 0.998. The LODs were determined as the concentrations corresponding to three times the noise signal (*n*=5) and the LOQs as the lowest calibration level of each compound. The corresponding values expressed as ng/mg of hair are also listed in Table 1.

The precision was examined at three different concentrations. Repeatability was evaluated by injecting three replicates of each concentration level in duplicate on the same day and under identical experimental conditions. Intermediate precision was assessed on five consecutive days by injecting three replicates of each concentration level each day. The results, presented in Table 1, are expressed as the %RSD of the peak areas. Recovery study was carried out by comparing the peak areas corresponding to pooled blank hair spiked with three different concentrations, with the peak areas obtained for standard solutions containing the same concentration levels, all analysed using the developed PLE/in-line SPE-CE method. The mean extraction yield values were calculated from three independent analyses. Relative recoveries greater than 80% were obtained (Table 1). As can be seen, the method proposed in the present

**Table 1.** Regression equations, repeatability and reproducibility values, LODs and relative recoveries obtained for hair samples by PLE/in-line SPE-CE-DAD.

| | Mephedrone | | 4-Methylephedrine | | MDPV | |
|---|---|---|---|---|---|---|
| | *Enantiomer 1* | *Enantiomer 2* | *Enantiomer 1* | *Enantiomer 2* | *Enantiomer 1* | *Enantiomer 2* |
| **Linearity (ng/mg)** | 0.05−5.00 | 0.05−5.00 | 0.05−5.00 | 0.05−5.00 | 0.40−10.00 | 0.40−10.00 |
| **Calibration curve** [a] | y=68.618x +2.8810 | y=68.643x +2.7105 | y=68.342x +2.7913 | y=68.493x +2.7439 | y=44.653x -6.3412 | y=45.006x -6.8286 |
| **$r^2$** | 0.9983 | 0.9986 | 0.9987 | 0.9986 | 0.9989 | 0.9982 |
| **LOD (ng/mg)** | 0.02 | 0.02 | 0.02 | 0.02 | 0.1 | 0.1 |
| **Repeatability (%RSD)** [b] | | | | | | |
| 0.5 ng/mg | 7.8 | 7.6 | 7.9 | 8.5 | 5.8 | 6.1 |
| 1.0 ng/mg | 6.1 | 5.8 | 6.6 | 6.1 | 5.3 | 5.4 |
| 4.0 ng/mg | 6.7 | 6.3 | 7.1 | 6.9 | 6.1 | 5.8 |
| **Reproducibility (%RSD)** [c] | | | | | | |
| 0.5 ng/mg | 9.4 | 8.1 | 8.7 | 7.6 | 6.9 | 9.4 |
| 1.0 ng/mg | 8.2 | 9.4 | 8.9 | 8.1 | 8.5 | 8.2 |
| 4.0 ng/mg | 8.3 | 8.9 | 7.9 | 7.5 | 6.8 | 7.1 |
| **Relative recoveries (%, n=3)** | | | | | | |
| 0.5 ng/mg | 91.2 | 87.5 | 85.5 | 82.3 | 81.3 | 91.2 |
| 1.0 ng/mg | 85.0 | 94.1 | 93.2 | 90.3 | 90.5 | 85.0 |
| 4.0 ng/mg | 91.2 | 92.6 | 92.7 | 94.5 | 84.8 | 84.6 |

[a] y: peak area value (mAU × seconds); x: concentration (ng/mg).
[b] intra-day analysis (%, n=6).
[c] inter-day analysis (%, n=15).

work provides satisfactory results in terms of recoveries and precision.

To date, only methods for the achiral determination of synthetic cathinones in the hair matrix have been reported [11, 14, 15]. For example, the method proposed by Hackett *et al.* [37], to determine several APs and cathinone designer drugs, including mephedrone and MDPV, in 10 mg of hair using a basic digestion (30 min) and SPE followed by an evaporation/reconstitution step for sample preparation, and LC-tandem MS analysis, recorded an LOD of 0.05 ng/mg of hair for all the analytes. Another study conducted on the same sample type (50 mg of hair),

using overnight incubation in a buffer solution and LLE followed by an analysis, gave an LOQ of 0.2 ng/mg of hair for mephedrone [7]. The method was successfully applied to hair from drug abusers and mephedrone was found at concentrations between 0.2 and 313.2 ng/mg of hair. The same LOQ value (0.2 ng/mg of hair) was obtained for mephedrone in 100 mg of hair by Gerace *et al.* [6] using overnight incubation in MeOH followed by an evaporation/reconstitution step and derivatization for sample preparation, and GC/MS for analysis.

In view of these data, despite that we used a relatively large amount of hair (100 mg), which may be considered as a drawback of this approach when sample availability is limited, the method proposed in this paper, which combines in-line SPE-CE with chiral separation, is faster, simpler and more cost-effective, as well as highly environmentally friendly. In addition, it can provide information of both enantiomers of a chiral drug in the hair matrix.

## 4. Concluding remarks

The present paper describes a simple and cheap methodology, using a non-sophisticated instrumentation, proper for determining the enantiomeric composition of evaporation/reconstitution step for sample preparation, and GC/MS cathinone drugs in hair, providing information of both drug enantiomers. Further work should focus on using this novel strategy for the identification and quantitative determination of these racemic drugs of abuse in hair specimens from drug abusers to evaluate the incorporation mechanisms of the cathinone drugs into hair.

### Acknowledgements

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UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES
Tatiana Baciu

## 3.1.4. Discussion of results

UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
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In this section, there is a discussion of the most relevant aspects of three methodologies based on the use of in-line SPE as a clean-up step and enrichment technique, in combination with CE-UV for the determination of different DOAs in hair samples.

**In the first study** (Section 3.1.1.), an in-line SPE-CE approach was satisfactorily developed and applied in conjunction with a previous sample pretreatment based on a digestion procedure in an acidic medium for determining cocaine and its major metabolite, benzoylecgonine, in hair samples.

For the optimization of the in-line SPE-CE preconcentration technique, a preliminary study was carried out to evaluate the performance of five commercial SPE materials. To be specific, one sorbent based on hydrophobic interactions (Oasis HLB), three mixed-mode sorbents (Oasis MCX, Bond Elut Plexa PCX, and LiChrolut TSC) based on hydrophobic interactions and cation exchange and one sorbent based on strong cation exchange (LiChrolut SCX) were tested. Cocaine and benzoylecgonine consist of a hydrophobic (benzene ring) and hydrophilic (tertiary amine) region.

To summarize, in the case of Oasis HLB, the pH of the sample was adjusted to 9.5, at which cocaine is uncharged and benzoylecgonine is zwitterionic so that their retention in the sorbent is favoured. As the elution solvent, methanol containing 2% acetic acid was used to positively charge the target compounds and facilitate their release from the sorbent.

In the case of Oasis MCX, Bond Elut Plexa PCX, LiChrolut TSC, and LiChrolut SCX, the pH of the sample was adjusted to 2.5 to have the analytes under study positively charged in order to exploit the ion exchange retention mechanism between them and the sorbents. As the elution solvent, methanol containing 2% ammonium hydroxide was used. Under these conditions, cocaine becomes neutral, whereas benzoylecgonine is negatively charged and both can be successfully released from the sorbents.

Figure 1 shows the electropherograms obtained with the sorbents examined. From this figure, the following facts can be observed: (i) the best responses in terms of peak area were obtained for cocaine with Oasis HLB, whereas, for benzoylecgonine, the best response was with both Oasis HLB and Oasis MCX; (ii) unsatisfactory results were achieved with Bond Elut Plexa PCX and LiChrolut TSC, even though these SPE

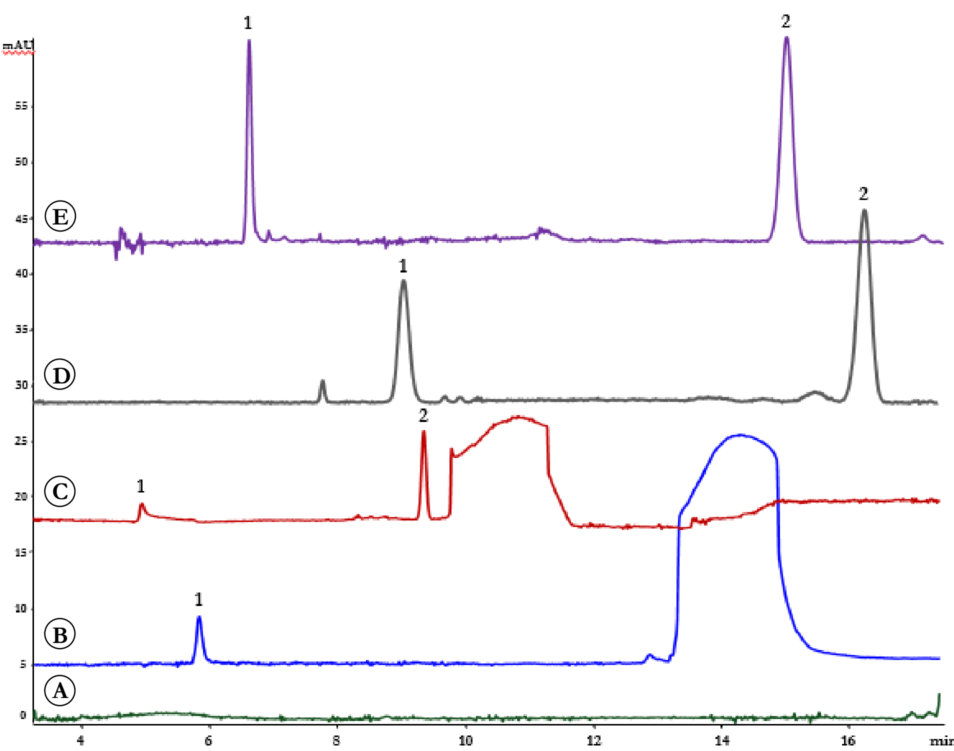


**Figure 1.** Selection of the SPE sorbent for the construction of the in-line SPE-CE device: (A) LiChrolut SCX, (B) LiChrolut TSC, (C) Bond Elut Plexa PCX, (D) Oasis MCX, (E) Oasis HLB. Other experimental conditions are reported in Subsection 3.1.1. Peak identification: 1, cocaine and 2, benzoylecgonine.

materials have similar chemical properties to Oasis MCX and, therefore, a similar retention to that achieved with the latter was initially expected; and, finally, (iii) no peaks were obtained with SCX.

From the above observations, it can be concluded that Oasis HLB and Oasis MCX clearly provided the best responses in terms of peak area. In the case of the Bond Elut Plexa PCX, LiChrolut TSC, and LiChrolut SCX, it seems that cocaine and benzoylecgonine interacted with the sorbents through hydrophobic interactions, which were more significant than ionic interactions. This was not initially expected as the analytes were in their cationic form at pH 2.5 and, therefore, the ionic interactions were expected to be more significant.

Moreover, when the Oasis MCX, Bond Elut Plexa PCX, LiChrolut TSC, and LiChrolut SCX sorbents were used, potential problems were experienced, such as current disruption and poor repeatability. This behaviour might be attributable to the incompatibility between the pH of the acidic BGE and the alkaline solvent used for the elution of the analytes from the sorbents. In such a scenario, despite the attempts to improve selectivity, it became evident that Oasis HLB was the most suitable sorbent for our purpose, so it was selected to enrich cocaine and benzoylecgonine.

As well as the selection of the kind of sorbent, another important parameter affecting the in-line preconcentration that should be considered is the sample pH. In the literature, different sample pH values have been reported when using Oasis HLB was used to extract DOAs. Good retention for off-line SPE has been reported at acidic pH [1], neutral pH [2, 3] and basic pH [4]. As such, there is no consensus regarding the best pH of the sample to be used, so the effect of the pH of the sample (2.5, 7.0 and 9.5) on the retention of cocaine and benzoylecgonine was explored under our experimental conditions to identify the optimal case. The best responses in terms of peak area were obtained at pH 9.5.

The volume of the elution solvent represents another key factor for in-line SPE-CE, as this volume is generally higher than the conventional hydrodynamic volumes introduced in CE. The injection time of the elution plug should be the minimum possible to guarantee the quantitative elution of the target analytes. If it is not correctly chosen, it can lead to lower peak efficiency and resolution, as well as problems with the stability of the current. In our case, the elution volume was evaluated by injecting the solvent at 50 mbar over different periods of time from 5 to 40 seconds, which corresponds to elution volumes between 6 and 47 nL (calculated using the Hagen-Poiseuille equation). An elution volume of 23 nL (50 mbar, 20 seconds) provided the best responses in terms of peak area without losses of efficiency and resolution. The solvent plug was then driven through the SPE microcartridge in order to elute the analytes from the sorbent before CE separation by introducing BGE solution at 50 mbar for 250 seconds, which corresponds to a capillary volume of 293 nL (15.1 cm of the capillary length). This is enough to ensure that the elution solvent is displaced out of the SPE device.

Moreover, it is usually stated that injecting large volumes of a sample in SPE represents the most straightforward way to increase sensitivity and thereby obtain lower LODs [5, 6]. The sample loading time was tested between 15 and 50 minutes

at 930 mbar, and it was observed that, above 40 minutes, the increase in sensitivity was not significant. To avoid prolonging the overall analysis time extensively, a sample loading time of 30 minutes was selected to validate the analytical method, which corresponds to about 40 μL of sample.

To extract cocaine and benzoylecgonine from the hair matrix, an overnight digestion was performed with easy-to-handle 0.1 M hydrochloric acid. The resulting extraction solutions were adjusted to pH 9.5 with an aqueous sodium hydroxide solution and then directly injected into the in-line SPE-CE-UV system for analysis. This fact enabled a considerable simplification of the sample treatment to be achieved compared to the current CE-based methods that include LLE procedures after the extraction, as already cited in earlier sections.

LODs of 0.02 ng/mg for cocaine and 0.10 ng/mg for benzoylecgonine were satisfactorily achieved using 50 mg of hair sample. However, the LOD concentration obtained for benzoylecgonine is slightly above the cut-off level in hair proposed by the Society of Hair Testing to identify (ab)use of cocaine [7]: 0.5 ng/mg and 0.05 ng/mg for cocaine and its metabolites, respectively. Despite this fact, the sensitivity acquired here is adequate for determining illicit drug (ab)use in therapeutic drug monitoring, drug rehabilitation programs, doping control, workplace and even forensic cases.

The relevance of our first study is based on its ability to provide excellent results without the need for sophisticated instrumentation. The extraction, in-line preconcentration, and electrophoretic separation procedures were satisfactorily combined altogether, resulting in a cheap and easily performed methodology suitable for hair drug testing. To explore its potential, it was used to determine the concentrations of the DOAs investigated in hair samples collected from patients undergoing a drug detoxification program, proving to be an excellent analytical tool for such a complex task. The findings in the hair samples of the drugs abusers were generally in line with the attempted detoxification and the data provided by them at the time of sampling.

The promising results accomplished here encouraged us to continue exploring the capabilities of the in-line SPE-CE for drug testing in hair samples. At this point, our attention was focused on developing methodologies that enabled the extraction process of the DOAs from the hair matrix to be made faster compared to the acidic

digestion previously used, as well as being suitable for combination with in-line SPE-CE-UV systems. One strategy in response to this issue is the use of PLE, and this extraction strategy was investigated and used in the two research works described below.

**In the second study** (Section 3.1.2.), an in-line SPE-CD-modified-CE method with a previous sample pretreatment procedure based on PLE was successfully developed for the detection and quantification of 6-acetylmorphine, benzoylecgonine, cocaine, codeine, morphine and the methadone enantiomers in hair samples.

At the beginning of the method development, it was observed that these compounds, with the exception of benzoylecgonine, are difficult to resolve using the conventional CE-UV because of their very similar charge to size ratios under acidic conditions. This limitation was circumvented satisfactorily here simply by adding an appropriate CD to the electrolyte solution. Specifically, the native α- and β-CD were tested, of which α-CD provided the best electrophoretic separation of the target drugs, although β-CD also proved effective for such a task. This excellent achievement clearly opens several possibilities for other analytes with similar chemical structures to be successfully resolved by conventional CE-UV, such as benzodiazepines.

As well as the benefit of significantly improving the resolution of 6-acetylmorphine, cocaine, codeine, morphine, and methadone, the use of α-CD, also successfully enabled one of the aims of this work to be achieved, namely the separation of the enantiomers of methadone, making it possible to separate both chiral and non-chiral DOAs within one analytical run. This ability represents promising progress toward developing more versatile and low-cost analytical methodologies compared to those based on techniques such as LC or GC, which usually require relative expensive chiral columns that are particularly suited to specific types of analytes [8].

With respect to the in-line SPE-CE preconcentration technique, Oasis HLB was the SPE sorbent used for the construction of the analyte concentrator device since our previous results obtained when working with this material were highly satisfactory (Section 3.1.1.). The sample pH was set at 9.1 based on the results obtained for similar compounds in the earlier research, as well as the $pK_a$ values of the analytes of interest (provided in Appendix II). Under these conditions, cocaine and methadone are in their non-ionic form, while the remaining DOAs are zwitterionic so that their retention in the sorbent is favoured. The elution volume was evaluated by introducing

a solvent plug for different periods of time from 5 to 30 seconds at 50 mbar, which corresponds to elution volumes between 5 and 30 nL. An elution volume of 24 nL (50 mbar, 25 seconds) provided the best responses in terms of peak area. The solvent plug was driven through the SPE sorbent by introducing BGE solution at 50 mbar for 250 seconds, which corresponds to a capillary volume of about 240 nL (12.4 cm of the capillary length). The sample loading time was tested between 10 and 40 minutes at 930 mbar. As a compromise between sensitivity and analysis time, a sample loading time of 30 minutes, which corresponds to about 32 μL of sample, was selected to validate the analytical method.

Another important achievement of this research work was the development of an efficient PLE protocol appropriate for the extraction of several DOAs from hair samples using an acidic aqueous solution as the extracting medium (namely, water adjusted to pH 2.0 with HCl 37%). Besides this ability, the extraction methodology developed here exhibits other significant benefits that would clearly give a further boost to this technique and promote its broader application for such tasks: (i) the hugely reduced time required for extraction compared to the conventional acidic digestion procedure used in our first study; and (ii) the possibility of directly injecting the hair extracts obtained from the PLE into the in-line SPE-CE-UV system for their analysis without the need for further sample handling. The latter significantly simplifies the sample preparation procedure compared to the existing LC-based methods that include an off-line SPE after the extraction of DOAs from hair by PLE [9, 10].

The overall time required for hair sample analysis including decontamination, PLE extraction, and in-line SPE-CE-UV analysis is about 2 hours, which may be considered reasonable time, taking into account the use of non-sophisticated instrumentation that is easy to handle. This fact makes this approach an attractive candidate for routine analysis of DOAs compared to those including conventional extraction methods, which, as already discussed, usually involve extensive processing periods.

Using aliquots of 100 mg of hair, the LODs achieved with UV detection (0.10-0.13 ng/mg) were generally in line with the cut-off concentrations proposed by the Society of Hair Testing for detecting these analytes in hair [7], except for morphine (LOD of 1.0 ng/mg). The organization recommends a cut-off level of 0.2 ng/mg in hair to

identify (ab)use of opiates. This means that the sensitivity achieved here for morphine is more suited for quantifying therapeutic levels.

Once validated, the method was successfully applied to determine the concentrations of the DOAs investigated in hair samples collected from patients undergoing a drug detoxification program, proving to be an excellent analytical tool for such a complex task. As in our previous research work, the findings in the hair samples of the drugs abusers were generally in line with the attempted detoxification and the data provided by them at the time of sampling.

**In the third study** (Section 3.1.3.), an analytical strategy based on the combination of PLE and in-line SPE-CD-modified-CE was satisfactorily established for the chiral determination of three synthetic cathinones in hair samples. The novel feature of this research is that it is the first CE-based methodology reported in the literature dealing with the chiral identification and quantification of such analytes in hair.

With regard to separation, several CDs have been reported in the literature as being useful for the enantiomeric resolution of synthetic cathinones by CE [11-13]. In view of this, four CDs were assessed under our experimental conditions to find out the most suitable for the intended purpose. Three natives (α-, β- and γ-CD) and one modified (sulphated-β-CD) were examined here, of which native β-CD provided the best chiral resolution for all the target drugs.

With respect to the preconcentration of the DOAs, as in our previous two research works, the in-line SPE-CE microcartridge consisted of a short length of a capillary of 2 mm packed with Oasis HLB sorbent. The sample pH was fixed at 10.0, based on our earlier findings which demonstrated that basic analytes are better retained on this SPE material at high pH levels, as well as the $pK_a$ values of the synthetic cathinones under study (provided in Appendix II). At this pH, the synthetic cathinones are uncharged and their retention in the sorbent is favoured. The elution volume was evaluated by introducing the organic solvent at 50 mbar over different periods of time from 5 to 30 seconds, which corresponds to elution volumes between 5 and 30 nL. An elution volume of 20 nL (50 mbar, 20 seconds) provided the best responses in terms of peak area without losses of efficiency and resolution. As well as in the previous works, in this study, the solvent plug was driven through the SPE sorbent by introducing BGE solution at 50 mbar for 250 seconds. Under these conditions, the volume of the capillary filled was about 240 nL, which corresponds to 12.4 cm of

the capillary length. The sample loading time was tested between 15 and 40 minutes at 930 mbar, and it was observed, that above 35 minutes, the increase in sensitivity was not significant. Therefore, a sample loading time of 40 minutes was selected as optimal, which corresponds to about 43 μL of sample.

Using the PLE method proposed, the extraction of the DOAs from the hair samples was completed in about 8 minutes using a basic aqueous solution as the extracting medium (namely, water adjusted to pH 10.0 with ammonium hydroxide 28%). The hair extracts obtained were suitable for direct injection into the in-line SPE-CE-UV system without needing any extra steps. The overall time required for hair sample analysis including decontamination, PLE extraction, and in-line SPE-CE-UV analysis is around 2 hours, which may be considered acceptable, taking into account its simplicity and ability to provide information on both enantiomers of a chiral drug in the hair matrix.

Cut-off levels for synthetic cathinones have not yet been reported in the literature. According to the recent guidelines of the European Workplace Drug Testing Society, the cut-off concentrations of synthetic cathinones in hair are expected to be comparable to those of amphetamines, since synthetic cathinones are structurally similar to amphetamines [14]. The Society of Hair Testing recommends a cut-off concentration of 0.2 ng/mg in hair to identify use of amphetamines [7]. In our case, using aliquots of 100 mg of hair, LODs of 0.02 ng/mg were achieved for the enantiomers of mephedrone and its metabolite, and 0.1 ng/mg for the enantiomers of 3,4-methylenedioxypyrovalerone, suggesting that the methodology presented here provides adequate sensitivity to determine the illicit (ab)use of synthetic cathinones satisfactorily.

When taking the results discussed in this section altogether, there is no doubt that in-line SPE-CE-UV has considerable potential for determining DOAs in hair samples. Here, it has successfully been demonstrated that a non-sophisticated analytical tool such as CE-UV is capable of dealing with complex tasks with great sensitivity. Certainly, these results will give a further boost to CE-UV and promote its broader application for hair drug testing for which this technique has scarcely been used to date.

Despite its great advantages, in-line SPE-CE-UV is not free from drawbacks, the most important probably being that it can often result in a time-consuming process,

because the sorbent material is held in a column or capillary connected to the separation capillary, and replacing it would require the cassette to be removed from the instrument every time. In addition, the SPE microcartridges need to be constructed by the user, since in-line SPE-CE devices are not yet commercially available, and this fact could affect the reproducibility of the CE methods. One promising strategy to improve these shortcomings is the use of magnetic adsorbents, and, in this respect, this strategy was used in the subsequent research described in the following sections.

Before starting the next sections, we would like to briefly discuss some of the future works that we propose. The in-line SPE-CE procedure developed here proved to be highly useful for improving the sensitivity of CE by enabling the injection of large volumes of sample by applying the maximal injection pressure of the instrument (930 mbar) over a relatively long period (30-40 minutes). Further research could focus on developing faster in-line SPE-CE strategies without sacrificing the sensitivity of the method. One strategy may be the application of a higher (external) pressure than the one commonly applied during the sample injection.

In relation to this, there are some preliminary results that are worth highlighting. In short:

1. In-line SPE microcartridges are not able to withstand injection pressures higher than 6,000 mbar.
2. An injection pressure of 6,000 mbar proved to be beneficial for speeding up the analysis time considerably. In the case of cocaine and benzoylecgonine, injecting the sample for 5 minutes at 6,000 mbar (42 μL) enabled the same sensitivity achieved by injecting the sample for 30 minutes at 930 mbar (40 μL).
3. Using an injection pressure of 6,000 mbar and sample loading times greater than 5 minutes, an increase in the responses in terms of peak area for cocaine and benzoylecgonine was observed, suggesting that the LODs obtained in our first research could be lowered further.

As well as this, different geometries of the in-line SPE-CE microcartridge could be investigated, since increasing the quantity of the SPE sorbent introduced in the microcartridge, the load capacity of the sample is greater and, consequently, lower LODs could be achieved. Moreover, future research is needed to evaluate different other SPE sorbents with the aim of improving selectivity.

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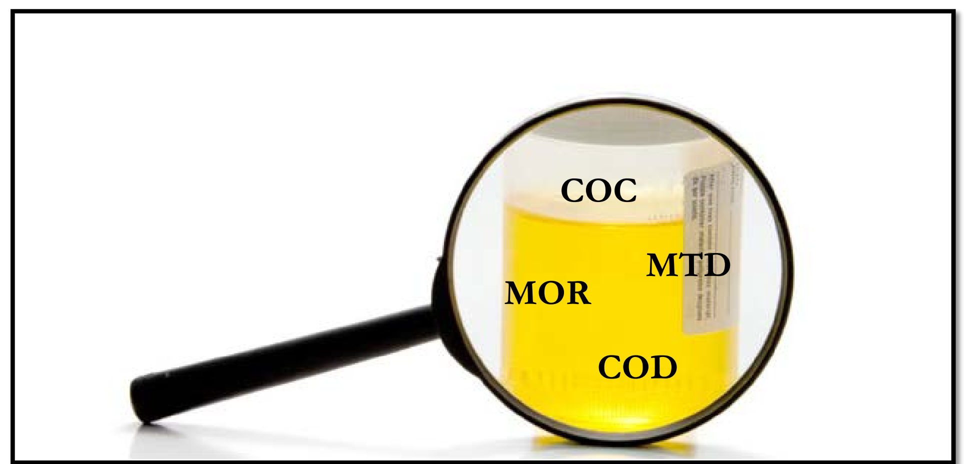
# 3.2. URINE TESTING FOR DRUGS OF ABUSE



In the previous sections, it has been successfully demonstrated that in-line SPE-CE-UV represents a powerful analytical tool for the determination of DOAs in hair samples. Particularly, the results obtained in terms of improving the sensitivity together with advantages such as being simple, low-cost and environmentally friendly will certainly contribute to expanding this methodology's application in the field of hair drug testing. Nevertheless, as mentioned above, there are still some issues that need to be addressed arising from the fact that in-line SPE-CE microcartridges need to be made in-house by the users, which can lead to irreproducibility.

In recent years, different approaches have been proposed in the literature for performing in-line extractions inside a CE capillary using magnetic particles as the adsorbents, in particular for the extraction of parabens and anti-inflammatory drugs [1] and immunoaffinity purification procedures [2-4]. Due to their unique properties, such particles may be manipulated inside a CE capillary simply by using external magnets located either side of the capillary inside the cassette. This peculiarity would enable the SPE material to be introduced directly into the separation capillary and then easily be pumped out after each analysis, instead of being permanently held in a separate capillary, as is the case in conventional in-line SPE-CE configurations.

With this idea in mind and considering that the combination of magnetic SPE in-line with CE has not been implemented to date for determining DOAs in biological samples, our efforts were directed towards establishing an automated in-line SPE-CE protocol using magnetic particles suitable for such a complex task to maximize knowledge of this promising strategy, as well as to explore the possible benefits of using it compared to conventional in-line SPE-CE configurations.

To this end, silica-coated $Fe_3O_4$ particles were prepared, functionalized with $C_{18}$, which is commonly used as a stationary phase in reversed-phase chromatography. Once characterized, the synthesized particles were loaded into the CE capillary and used to preconcentrate cocaine, codeine, methadone and morphine from urine samples prior to analysis. For trapping the magnetic particles inside the CE capillary, an in-house holder was used containing two permanent neodymium iron boron magnets. Several parameters affecting the in-line preconcentration were evaluated, such as the formation, holding and removal of the magnetic plug, amount of the

magnetic sorbent trapped inside the capillary, sample loading time and elution conditions.

As in our previous works, the method described in this section was validated in accordance with the guidelines of the Scientific Working Group for Forensic Toxicology and Society of Toxicological and Forensic Chemistry. Linearity, repeatability, reproducibility, LOD, LOQ and relative recoveries were evaluated using pooled drug-free urine samples spiked with the studied analytes. Once validated, the method was successfully applied to the analysis of the target DOAs in urine from drug abusers that were following a drug rehabilitation program.

The paper discussing the results of the present study was published in the journal Electrophoresis 37 (2016) 1232-1244.

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### *3.2.1. Capillary electrophoresis combined in-line with solid-phase extraction using magnetic particles as new adsorbents for the determination of drugs of abuse in human urine*

UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES
Tatiana Baciu

# CAPILLARY ELECTROPHORESIS COMBINED IN-LINE WITH SOLID-PHASE EXTRACTION USING MAGNETIC PARTICLES AS NEW ADSORBENTS FOR THE DETERMINATION OF DRUGS OF ABUSE IN HUMAN URINE

**Tatiana Baciu[1], Francesc Borrull[1], Christian Neusüß[2], Carme Aguilar[*,1] and Marta Calull[1]**

[1]Department of Analytical Chemistry and Organic Chemistry, Rovira i Virgili University, Campus Sescelades, Marcel·lí Domingo, 1, 43007 Tarragona, Spain

[2]Department of Chemistry, Aalen University, Aalen, Germany

## Abstract

A simple approach is presented based on the in-line coupling between magnetic particles-based SPE and CE. Silica-coated iron oxide particles functionalized with $C_{18}$ were successfully synthesized and used as a reverse-phase sorbent for in-line SPE-CE. Magnets were used to locally immobilize these sorbents inside the capillary. Four drugs of abuse were preconcentrated and determined in urine samples using the developed method with a simple pretreatment procedure based on LLE. Several parameters affecting the preconcentration were evaluated. The obtained results show that this strategy enhanced detection sensitivity in the range of 125–700-fold compared with CE without preconcentration. The developed method provides LODs (S/N=3) for standard samples in the range of 0.5–20 ng/mL with satisfactory analytical precision, in both intraday and day-to-day experiments (RSDs, 20%). The LODs (S/N=3) reached for urine samples were in the range of 20−50 ng/mL. Relative recoveries greater than 75.9% were obtained. The established method has been applied to the analysis of drugs of abuse in urine samples from drug abusers.

***Keywords****: bioanalysis/capillary electrophoresis/drugs of abuse/in-line solid-phase extraction/magnetic particles*

## 1. Introduction

Nowadays, with its high efficiency and resolution, possibility of automation, minimal consumption of reagents, and low sample volume for analysis, as well as its low cost, CE is a well-established analytical separation technique that has been successfully applied to determine a broad range of drugs of abuse in different types of matrices [1, 2]. However, in spite of these great benefits, it is well-known that CE suffers from poor concentration sensitivity, especially when DAD is used. To overcome this problem, several strategies have been developed, among which, the in-line coupling between SPE and CE has proven to be highly useful for improving the sensitivity of CE in the analysis of different kind of compounds, including drugs of abuse [3,4].

In in-line SPE-CE, an analyte concentrator is synthesized or placed as a short column near the inlet end of the CE capillary [5,6]. To do so, different set-ups have been reported in the literature, of which the most commonly used is the system based on the use of small packed beads, with the most widely employed sorbents being Oasis HLB, Oasis MCX, and $C_{18}$ [3, 7, 8]. In this configuration, the SPE material can be retained by means of two frits but, over time, their use has become less advisable due to issues with performance arising from several disadvantages discussed elsewhere [9]. In this respect, fritless designs using sorbent particles with a size larger than the ID of the CE capillary, in order to keep the particles in place, have been successfully developed and applied to improve the sensitivity of CE in the analysis of different types of analytes [3, 7, 8, 10].

To date, fritless in-line SPE-CE devices are not yet commercially available and need to be constructed by the user, a fact that may be considered as a drawback of this approach, since the reproducibility of the CE methods may be affected. In this context, magnetic SPE (MSPE) using magnetic particles (MPs) as solid-phase supports may represent an interesting alternative as, due to their magnetic properties, these materials may easily be manipulated inside a CE capillary just by the application of a magnetic field. As a result, MPs represent an easier and faster way to create a concentrator inside the CE capillary with the possibility of renewing the magnetic adsorbents simply by refilling the capillary with a fresh plug of MPs.

Over the last few years, different types of MPs have been investigated and successfully used in sample preparation, of which iron oxides like

magnetite ($Fe_3O_4$) are the most popular, due to their low price and toxicity. For analytical purposes, these iron particles have always been modified and functionalized with different materials to improve their stability and introduce new surface properties. A series of substances have been used for this task, such as silica, $C_{18}$, carbon, surfactants, polymers, ionic liquids, etc. Several reviews have summarized and thoroughly discussed the preparation, structure, properties, and applications of the functionalized MPs as new adsorbents for sample preparation and preconcentration of different organic and inorganic compounds [11–13].

In this context, several analytical procedures combining MSPE with CE have recently been reported [14–19]. Some of the proposed methods have demonstrated that functionalized MPs can be successfully exploited in the in-line modality for sample preparation and preconcentration of target compounds prior to their separation by CE [16–19]. To do so, functionalized MPs were trapped inside the CE capillary near the inlet end using permanent magnets placed inside the cassette. Tennico and Remcho [16] used $C_{18}$-functionalized silica $Fe_3O_4$ ($C_{18}$-FS-$Fe_3O_4$) particles for the in-line extraction of several parabens and nonsteroidal anti-inflammatory drugs prior to their separation by CE. However, only partial automation of the extraction process inside the CE capillary has been achieved, since the MSPE was performed outside, i.e. the functionalized MPs were added to the sample, mixed, and then injected into the CE system and retained by the magnets. To date, fully automated approaches using MPs in-line with CE have been implemented just for immunoaffinity purification procedures using MPs coated with appropriate antibodies immobilized inside the CE capillary as immunosupport for extraction and isolation of proteins such as α-lactalbumin [17], α-lactoglobulin [17, 18], or α-1 acid glycoprotein [19], prior to separation by CE.

The aim of the present study was to develop a cheap, simple, environmentally friendly and fully automated method for the analysis of four commonly abused drugs by means of the in-line coupling between MSPE and CE. The established method has been applied to the analysis of drugs of abuse in urine samples from drug abusers.

## 2. Materials and methods

### 2.1. Reagents and standards

All reagents used were of analytical-reagent grade. Ultrapure reagent water purified by a Milli-Q gradient system (Milli-pore, Bedford, MA, USA) was used throughout this work. Cocaine (COC), codeine (COD), methadone (MTD), and morphine (MOR) were purchased from LGC Standards S.L.U. (Barcelona, Spain). Acetone, acetonitrile, dichloro-methane, 2-propanol, methanol (MeOH), and toluene were HPLC grade and were acquired from J.T. Baker (Deventer, the Netherlands). Ammonium hydroxide (28%), cube-shaped $Fe_3O_4$ particles (97%, 50−100 nm), formic acid, dimethylformamide (99.8%), tetraethyl orthosilicate (99.0%), trimethoxyoctadecylsilane (90%), and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich (MO, USA).

Stock standard solutions of 1,000 mg/mL for each compound were prepared in MeOH and stored at -20ºC. From these standard solutions, working standard solutions of the mixture of all the compounds at a concentration of 100 mg/mL were prepared weekly by diluting in Milli-Q water and stored at 4ºC. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard stock solution in ultrapure water.

The working aqueous suspension of the MPs was prepared daily by placing 3 mg of the synthesized MPs into a CE glass vial of 2 mL containing 1 mL of Milli-Q water.

### 2.2. Instrumentation

The electrophoretic system was an Agilent 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a DAD and an external pressure source. The analytes were detected at 200 nm. Bare fused-silica capillary with an i.d. of 50 mm was purchased from Polymicro Technologies (Barcelona, Spain). The capillary chamber was set at 25ºC for all the experiments. For pH measurements, a Lab pH-meter Basic 20+ (Crison, Barcelona, Spain) was used.

For trapping MPs inside a CE capillary, two permanent neodymium iron boron (NdFeB) magnets are usually used [17–19]. These materials are available in different shapes, sizes, and grades. For our application, two disk-shaped Nd-FeB magnets were chosen that were 6 mm in diameter and 3 mm thick, grade N45 and nominal energy product of 43− 45 Megagauss Oersteds (Webcraft GmbH, Gottmadingen, Germany).

Microscopic visualizations were carried out using a stereo microscope (Euromex Microscopen B.V., Arnhem, The Netherlands) at 10× magnification.

### 2.3. Synthesis and characterization of the $C_{18}$-FS-$Fe_3O_4$ particles

Initially, silica-coated $Fe_3O_4$ particles were prepared following the Stöber method [20]. To do so, 1 g of cube-shaped $Fe_3O_4$ nanoparticles with average diameter of 75 nm was dispersed in 100 mL of 2-propanol/Milli-Q water (90:10, v/v) under $N_2$ (UHP grade) flow while being stirred. After adjusting the pH of the solution to 9 with 1.5 M ammonium hydroxide, 5 mL of tetraethyl orthosilicate was added dropwise. Stirring continued at room temperature for 16 h, followed by heating to 50ºC for an additional 8 h. The resulting silica-coated MPs were washed with Milli-Q water, 2-propanol, and then vacuum dried overnight.

Next, surface modification with $C_{18}$ of the silica-coated MPs obtained from the previous step was carried out using the method reported in the literature [16]. To do so, the obtained silica-coated MPs (0.5 g) were dispersed in 100 mL of dimethyl-formamide/toluene (80:20, v/v) under $N_2$ flow while being stirred. Then, 10 mL of trimethoxy-octadecylsilane was added dropwise and the solution was left to be stirred for 24 h at room temperature. Finally, the product was washed with toluene for three times and then vacuum dried overnight.

The resulting $C_{18}$-FS-$Fe_3O_4$ particles were characterized by Fourier transform infrared (FT-IR) spectroscopy using a Jasco FT/IR-600 Plus ATR Specac Golden Gate spectrometer, transmission electron microscopy (TEM, JEOL model 1011 with 0.2 nm resolution) and scanning electron microscopy (SEM, JEOL model JSM-6400 with 3.5 nm resolution).

### 2.4. Urine collection and preparation

Urine samples were obtained from several patients undergoing MTD maintenance treatment in the CAS Tarragona Drug Addiction Monitoring and Support Centre (GIPPS Health) in Tarragona, Spain. Blank urine samples were obtained from non-addicted volunteers. All urine samples were collected in polypropylene tubes and stored at -20ºC until analysis. For method validation in urine, was used pooled urine prepared by mixing urine from several volunteers.

Before the in-line MSPE-CE analysis, the urine samples, spiked, and from drug abusers, were alkalized to pH 9.5 with ammonium hydroxide and extracted by LLE using the method described by Botello *et al.* [21] with modifications. In short, the extraction procedure was carried out by adding 500 mL of dichloro-methane/2-propanol (9:1, v/v) to 500 mL of spiked blank urine samples. After vortex mixing for 1 min, the samples were centrifuged for 10 min at 9,000 rpm (Hettich Zentrifugen, Germany). The organic phase containing the analytes was then transferred to an Eppendorf tube and a second extraction of the urine samples was performed by adding another 500 mL of dichloro-methane/2-propanol (9:1, v/v) and repeating the procedure previously described. Finally, the two organic phases were combined and then evaporated to dryness under a gentle stream of $N_2$. The residue was reconstituted with 500 mL of Milli-Q water adjusted to pH 9.0 with 28% ammonium hydroxide, filtered through a 0.45 mm nylon filter and then transferred to a micro vial for analysis.

## 2.5. CE separation without in-line preconcentration

The CE separation was performed on a fused-silica capillary with a total length of 80 cm (71.5 cm effective length) with an i.d. of 50 mm and an o.d. of 360 mm. The separation voltage was 15 kV (positive polarity). The BGE consisted of an aqueous solution of 40 mM ammonium acetate adjusted with 28% ammonium hydroxide to pH 8.7. Before the first use, the capillary was conditioned with 1 M NaOH for 40 min and Milli-Q water for 10 min at 930 mbar. At the beginning of each day, the capillary was rinsed with 0.1 M NaOH for 5 min and Milli-Q water for 5 min at 930 mbar. The capillary was then conditioned by flushing with BGE for 10 min at 930 mbar. Between runs, the capillary was conditioned with 0.1 M NaOH for 4 min, Milli-Q water for 4 min and running buffer for 4 min at 930 mbar. Standard samples were injected using hydrodynamic injection, applying a pressure of 50 mbar for 10 s.

## 2.6. In-line MSPE-CE procedure

Prior to the sample injection, the vial containing the magnetic $C_{18}$ sorbents in Milli-Q water (3 mg/mL) was set into the CE instrument and $N_2$ at 2 bars for 3 min was bubbled

through it in order to obtain a suspension of the particles. Afterwards, the obtained suspension was injected at a pressure of 930 mbar for 3 min. In this step, the $C_{18}$-FS-$Fe_3O_4$ particles are trapped by the magnetic field. More details about the magnet arrangement used for trapping the particles inside the capillary are provided in Section 3.2.2.

The capillary with the $C_{18}$-FS-$Fe_3O_4$ particles was then conditioned at 930 mbar with MeOH for 2 min and Milli-Q water (adjusted to pH 9.0 with 28% ammonium hydroxide) for 2 min. This step also allows the removal of the untrapped MPs. Once the functionalized particles were activated, the standard solutions or samples (adjusted to pH 9.0 with 28% ammonium hydroxide) were injected at 100 mbar for 30 min. Sample clean-up was then performed with BGE solution by applying 930 mbar for 2 min. This step allows the removal of untrapped molecules and ensures adequate starting conditions for the separation. Next, the retained analytes were eluted by injecting a plug of MeOH with 2.0% of formic acid at 50 mbar for 20 s. The elution plug was then pushed from the MSPE-CE system with the BGE at 50 mbar for 4 min. Lastly, a voltage of 15 kV was applied for the electrophoretic separation of the analytes.

Prior to the next run, the capillary was flushed with Milli-Q water at 12 bars for 5 min in order to remove the MPs and was then filled with $N_2$ by applying 2 bars for 2 min before carrying out the bubbling of the MPs as mentioned above.

## 3. Results and discussion

This section describes the optimization and validation process of the proposed in-line MSPE-CE for determining COC, COD, MTD, and MOR, as well as the results obtained when urine samples of four drug abusers were analyzed.

### 3.1. Optimization of CZE separation

According to the literature, COC, COD, and MTD have a p*Ka* of 8.6, 8.2, and 8.9, respectively, while, in the case of MOR, the p*Ka* values are 8.2 and 9.8 [22]. In this context, the literature reports that CE separations of opiates and COC can either be performed in acidic or basic BGEs since, under these conditions, these compounds are either fully or partially protonated, respectively [1, 2, 23].

As is well-known, in in-line SPE-CE systems, the sorbent is fully integrated into the CE instrument, so that the preconcentration and separation of the analytes is performed in the same capillary. To do so, after the analytes

are trapped on the SPE material, the capillary is filled with BGE just before separation to ensure adequate starting conditions. Lastly, the analytes are eluted from the SPE sorbent by introducing a plug of a solvent and then separated by CE. In view of this, the influence of the pH of the BGE on the retention of the compounds should be taken into account when this is chosen because undesired desorption from the SPE material may occur.

In our work, according to the pK*a* values of the target compounds, their retention in the $C_{18}$ sorbent would be favored at basic pH values. With this in mind, in order to optimize the separation of the drugs by CZE, an alkaline aqueous solution of ammonium acetate was selected as the CE buffer. This choice was based on the existing literature regarding the electrophoretic separation of different drugs of abuse [24, 25].

At the beginning of this study, a BGE was used consisting of an aqueous solution of 20 mM ammonium acetate adjusted with 28% ammonium hydroxide to pH 8.0 and an applied voltage of 30 kV. Under these conditions, as shown in Figure 1A, two peaks were obtained, indicating that the four analytes of interest were not completely resolved. Initially, it was suspected that the applied voltage was too high, leading to an excessively short analysis time and overlapping peaks. In this context, the effect of the applied voltage on the separation was firstly investigated at different values: 10, 15, 20, and 25 kV. The optimal separation voltage was found to be 15 kV (Figure 1A). At this applied voltage, satisfactory separation of COC and MTD was achieved, although separation of COD and MOR did not occur.

As mentioned above, in the first stage of our separation, the BGE was adjusted to pH 8.0. At this pH, taking into account that we were working very close to the pK*a* values of the target analytes, the separation achieved was not so bad, although COD and MOR coeluted (Figure 1A). This was attributed to the fact that COD and MOR have similar molecular sizes and their tertiary amino group has a pK*a* of 8.2 for both compounds. In consequence, the two analytes seem to have similar electrophoretic mobilities at pH 8.0. In this respect, to improve the separation between COD and MOR, considering the pK*a* values of all the examined drugs, different pH values of the BGE were tested within the range of 8–9. Figure 1B illustrates the electropherograms obtained. A pH of 8.7 was found to be the optimal for the desired analysis. At this pH, MTD is found close to its pK*a*, but below,

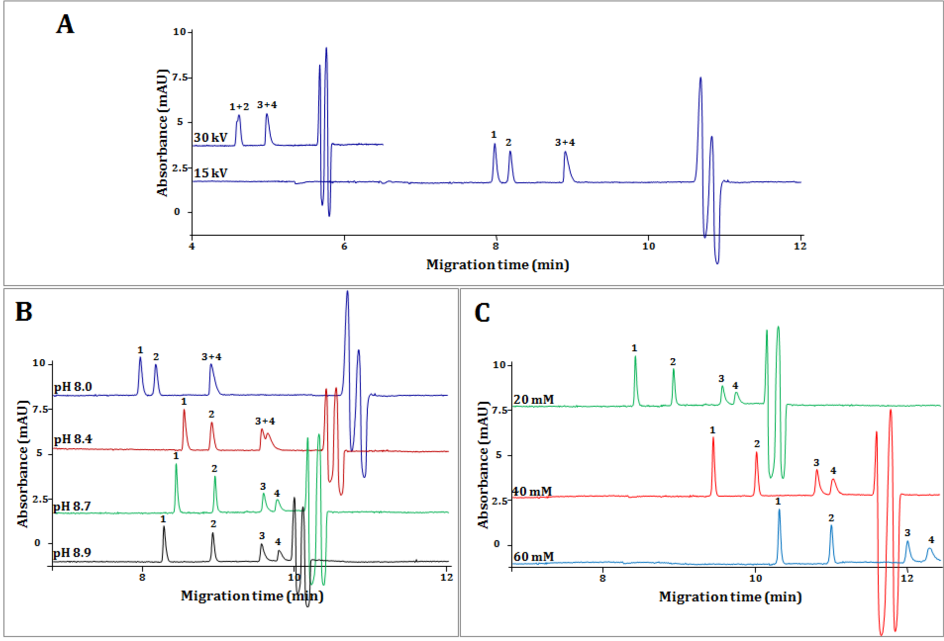


**Figure 1.** Effect of applied voltage (A), effect of pH value of the BGE (B) and effect of concentration of the BGE (C) on the CZE separation of the target compounds. Experimental conditions are reported in the text. The concentration of the analytes in standard samples was 30 mg/mL. Peak assignments: 1, MTD; 2, COC; 3, COD, and 4, MOR.

so it still positively ionized, while COC and COD would in principle be neutral. However, according to our results (Figure 1B), it seems that they were still protonated and this fact allowed their separation by CZE. MOR has an isoelectric point of 9.0, which means that, at pH 9.0, the positive and negative charges are both present in equal amounts and MOR could be considered as neutral while, below this pH, it could be considered that the positive charge predominates. This fact explains separation of MOR by CZE at pH 8.7.

Finally, the BGE concentration was varied from 20 to 60 mM to explore the effect that this parameter may have on our separation. Figure 1C shows the electropherograms obtained. Regarding the resolution, no significant improvements were detected. However, an aqueous solution of 40 mM ammonium acetate provided slightly better results in terms of sensitivity (peak height), so 40 mM was selected as the optimal BGE concentration.

## 3.2. Preconcentration by in-line MSPE-CE

As stated in the introduction, Tennico and Remcho [16] have successfully shown the feasibility and utility of performing in-line extractions using $C_{18}$-FS-$Fe_3O_4$ particles in a CE capillary.

With this in mind, one of the purposes of our study was to prepare $C_{18}$-FS-$Fe_3O_4$ particles and then use these materials as the adsorbent for the construction of an in-line SPE concentrator in CE to be applied in the analysis of drugs of abuse, particularly COC, COD, MTD, and MOR, to explore the possible benefits of this new approach compared to the conventional in-line SPE-CE configurations mentioned. In addition, taking into consideration the retention mechanism of the $C_{18}$ materials, drugs of abuse can also be effectively enriched by the $C_{18}$-alkyl chains through hydrophobic interactions, since although they are polar, their molecules contain both hydrophilic and hydrophobic parts.

### 3.2.1. Characterization of the magnetic $C_{18}$ particles

The synthesized $C_{18}$-FS-$Fe_3O_4$ particles were characterized by SEM, TEM, and FT-IR spectroscopy. As it can be observed in Figure 2A and B, TEM and SEM images show that the $Fe_3O_4$ nanoparticles (dark spots) are embedded in the silica matrix (gray layer) and the resulting MPs have a quasi-cubic morphology with sizes in the range of 200−300 nm.

Figure 2C depicts the IR spectrum of the $C_{18}$-FS-$Fe_3O_4$ particles. An absorption peak around 600 $cm^{-1}$ is the characteristic absorption to an Fe−O bond, confirming the presence of $Fe_3O_4$ nanoparticles. The adsorption peaks at 1,045 and 792 $cm^{-1}$ corresponds to the asymmetric and symmetric stretching vibration of Si−O−Si, indicating that the $Fe_3O_4$ nanoparticles were successfully coated with a silica layer. The peak at 960 $cm^{-1}$ can be attributed to Si−OH stretching, while the peak at 1,648 $cm^{-1}$ may be due to the bending vibration of water molecules.

In addition, as shown in Figure 3A, in the absence of an external magnetic field, the $C_{18}$-FS-$Fe_3O_4$ particles are well dispersed in aqueous solution, while, under the influence of an external magnetic field, these are immediately attracted to the side of the vial where the magnet is placed leaving a clear and transparent aqueous solution. This demonstration clearly suggests that the magnetic $C_{18}$ particles prepared in our laboratory are suitable for magnetic applications, such as MSPE.

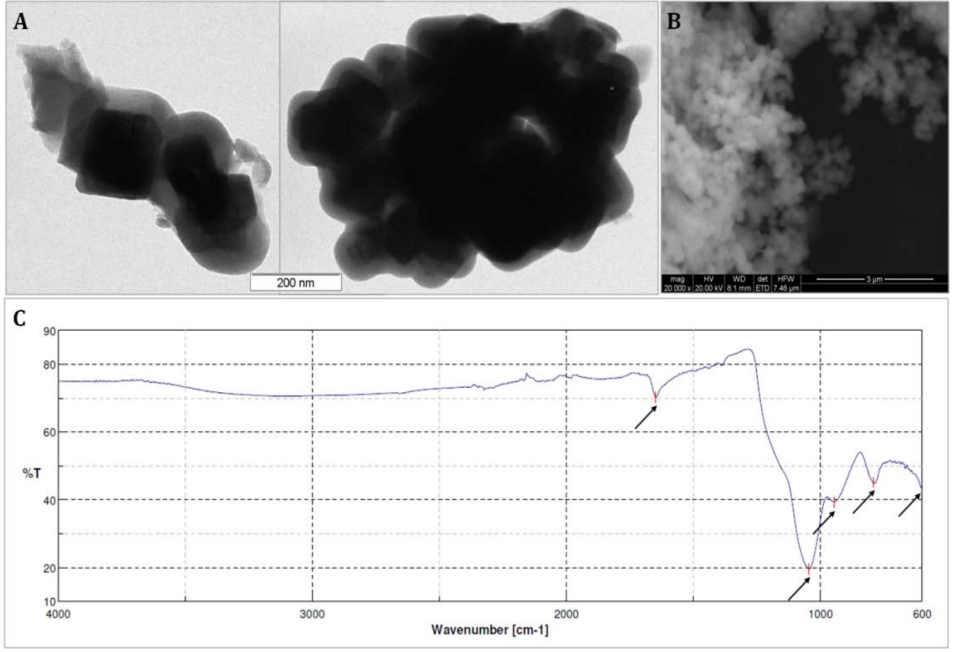


**Figure 2.** TEM image (A), SEM image (B), and FT-IR spectrum (C) of the $C_{18}$-FS-$Fe_3O_4$ particles synthesized in our laboratory.

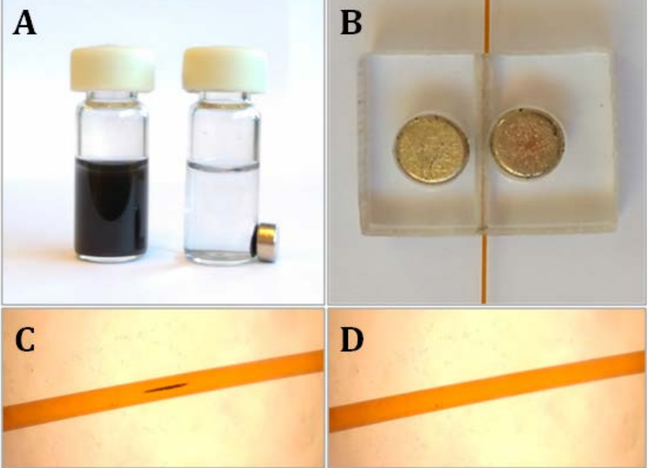


**Figure 3.** Photographs of the dispersion and separation process (A), homemade holding containing the magnets (B), $C_{18}$-FS-$Fe_3O_4$ particles trapped inside the capillary (C), and capillary after the removal of the MPs (D).

### 3.2.2. Magnet positioning

To retain the $C_{18}$-FS-$Fe_3O_4$ particles inside the capillary, the magnets were placed in attraction configuration with spacing of 1 mm around the capillary using a Plexiglas holder. This holder, which is illustrated in Figure 3B, was constructed in our laboratory based on the device's design used by Morales-Cid *et al.* [19] for on-line immunoaffinity CE with magnetic supports. The holder containing the magnets was set at a distance of 10 cm from the inlet end of the capillary.

### 3.2.2. Formation, holding, and removal of the magnetic plug

Before using the functionalized MPs as the adsorbent for the in-line MSPE-CE of COC, COD, MTD, and MOR, a series of preliminary tests were performed to get some idea of the behavior of our $C_{18}$-FS-$Fe_3O_4$ particles inside a 50 mm fused-silica capillary.

To start, an aqueous suspension of the MPs (3 mg/mL) was injected at 930 mbar for different periods of time from 1 to 5 min and then microscopic visualizations were carried out. As reported by several authors, MPs in suspension tend to sediment after a while, which may induce bad reproducibility, and also an excessive amount of MPs trapped inside the capillary may result in blockage and/or generate current instability [18, 19]. It was therefore decided to use no longer than 5 min for loading the MPs inside the CE system. As an example, Figure 3C shows a photo of a microscopic view of the capillary. As can be seen from the figure, a dense plug was formed inside the capillary where the magnets were placed, proving that our $C_{18}$-FS-$Fe_3O_4$ particles were successfully trapped inside a small section of the capillary by attraction to the magnetic field supplied by the NdFeB magnets used.

These experiments were repeated the same day and also on three different days. Each time, the length of the plug formed inside the capillary was measured using a ruler and, although qualitatively, was observed that the plugs generated were similar.

Next, it was important to ascertain which pressure could be applied to remove the MPs from the capillary without needing to take the magnets out of the cassette, in order to simplify the process of unloading the MPs at the end of each run. To do so, after the MPs were loaded and trapped, the capillary was flushed with Milli-Q water at different pressures higher than 2 bars during different periods of time and then microscopic visualizations were carried out. In our

set-up, it was observed that the $C_{18}$-FS-$Fe_3O_4$ particles were removed when a pressure of 12 bars was applied during 5 min (Figure 3D), so these conditions were used for the following experiments. The use of such a high pressure suggests that the magnets used are strong enough to hold the particles in their place during the injection and rinsing steps at 930 mbar.

### 3.2.4. Sample pH

Taking into account the lipophilic phase of the $C_{18}$ sorbents, the neutral forms of the target compounds are expected to be easily retained on our $C_{18}$-FS-$Fe_3O_4$ particles. In this respect the pH of the sample was set at 9.0, mainly because of MOR, which, at this pH, may be considered as neutral, as discussed above. Regarding the other drugs, based on their p*Ka* values, at pH 9.0, they should be uncharged.

### 3.2.5. Effect of amount of MPs trapped inside the capillary

To capture enough amount of $C_{18}$-FS-$Fe_3O_4$ particles inside the capillary for performing an efficient preconcentration of the target compounds, the injection time of the MPs suspended in Milli-Q water (3 mg/mL) was evaluated. The study was carried out by loading a suspension of the MPs at 930 mbar for different periods of time from 1 to 5 min, followed by the injection of a standard solution containing the analytes at a concentration of 1,000 ng/mL at 930 mbar for 10 min. Other experimental conditions were the same as those described in Section 2.6.

Three replicates of each studied injection time were performed by repetitive application of the entire in-line MSPE-CE process (i.e. a new plug of MPs was generated inside the capillary each time) and the RSD values obtained are listed in Table 1. According to the data provided in this table, for injection times lower than 3 min, high RSD values were obtained, indicating the existence of a great variation. An explanation may be that as a smaller amount of MPs has been loaded, the plug generated inside the capillary is less solid and consequently less stable during the analysis. In contrast, for the injection time of 3 min and higher, satisfactory RSD values were obtained (<12.0%), suggesting that the MPs formed a more stable and robust plug inside the capillary.

Moreover, as shown in Figure 4A, in general, the peak areas increased with the injection time up to 3 min, while, for higher injection times, the peak areas remained relatively

**Table 1.** RSD values of peak area (%, *n*=3) obtained for the target compounds at the different injection time of the magnetic $C_{18}$ particles studied.

| | MTD | COC | COD | MOR |
|---|---|---|---|---|
| 1 min | 7,9 | 31,1 | 22,5 | 41,3 |
| 2 min | 3,7 | 16,9 | 14,9 | 19,0 |
| 3 min | 2,7 | 9,2 | 11,2 | 10,6 |
| 4 min | 2,8 | 9,7 | 10,8 | 11,3 |
| 5 min | 2,8 | 9,7 | 11,7 | 10,8 |

constant, so this injection time was chosen for further studies, which provides a stable current at the same time.

### 3.2.6. Effect of desorption conditions

To obtain high sensitivity in terms of peak area, desorption conditions for MSPE were also optimized. In the initial phase of the method development, pure MeOH was used as the eluent, based on previously published studies in which this organic solvent was used to elute different compounds from $C_{18}$ magnetic sorbents [26, 27]. However, the addition of an acid to the elution solvent can significantly enhance the desorption efficiency as, under these conditions, COC, COD, MTD, and MOR are positively charged and, in this way, more poorly retained by the $C_{18}$ sorbent. With this in mind, the formic acid content in the MeOH was investigated from 0.0% to 2.0%. As shown in Figure 4B, with the increase in the acid content, the peak areas increased, up to a formic acid content of 2.0%. In the case of COC and COD, a formic acid content of over 1.0% contributed little to the increase of the peak area, in this way, it was decided not to test formic acid contents over 2.0%. Finally, a formic acid content of 2.0% was selected for the following experiments.

Meanwhile, acetone and acetonitrile have also been reported in the literature as suitable eluents for desorption of analytes from $C_{18}$ magnetic sorbents [28, 29]. In view of this, acetone and acetonitrile were also investigated, both containing 2.0% of formic acid, and then compared with 2.0% formic acid content in MeOH. According to the results, illustrated in Figure 4C, the peak areas increased when MeOH containing 2.0% of formic acid was used, so this solution was selected as the optimal solvent.

Finally, the elution volume was studied by introducing the optimal solvent with a hydrodynamic injection

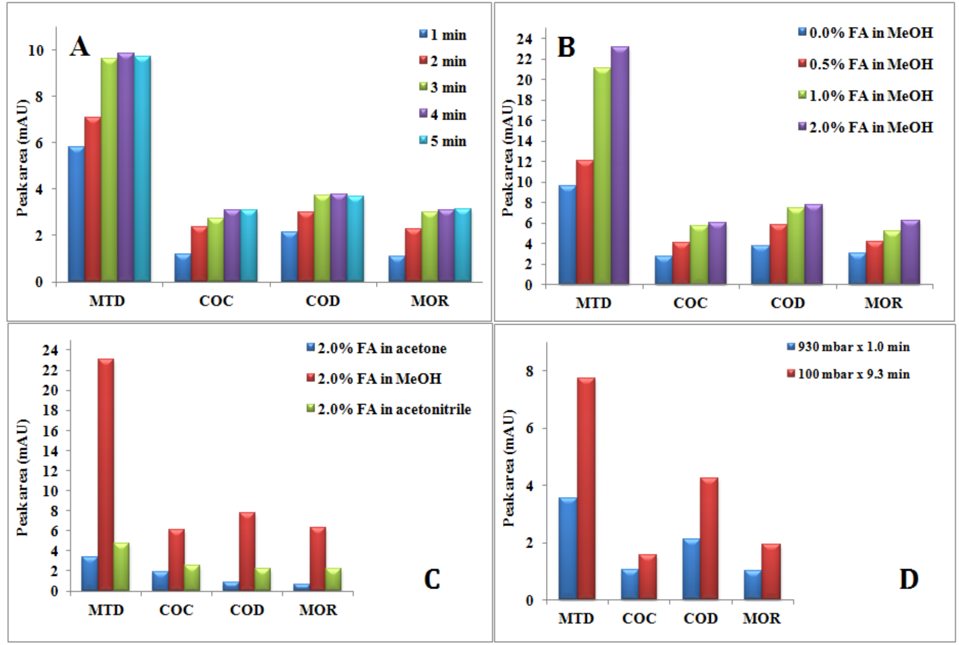


**Figure 4.** Effect of amount of MPs trapped inside the capillary (A), effect of formic acid (FA) content in MeOH (B), effect of type of organic solvent (C), and effect of sample injection pressure (D) on the peak area of the analytes by in-line MSPE-CE. Experimental conditions are reported in the text. The concentration of the analytes in standard samples was 1,000 ng/mL.

for different periods of time from 20 to 40 s at 50 mbar. In terms of peak area, no significant differences were obtained, probably because a plug of 20 s is enough to completely desorb all of the target compounds. Therefore, 20 s was selected as the elution time for further studies, which corresponds to about 20 nL (calculated using the Poiseuille equation) of organic solvent needed for eluting the four illicit drugs. This fact makes the proposed method in the present work an attractive, environmentally sustainable analytical tool because a very small amount of organic solvent is employed.

### 3.2.7. Effect of sample loading conditions

In a first instance, the use of an injection pressure of 930 mbar and of 100 mbar was evaluated with the purpose of comparing the effect of loading the sample at both high and low pressure, respectively. To do so, the same volume of sample should be injected in both cases. In this context, standard samples containing the drugs

at a concentration of 1,000 ng/mL were injected, firstly at 930 mbar for 1 min and then secondly, at 100 mbar for 9.3 min. Other experimental conditions were the same as those described in Section 2.6.

Three replicates of each studied injection pressure were performed by repetitive application of the entire in-line MSPE-CE process and satisfactory RSD values were obtained, in the range of 3.0−10.8%. Moreover, as shown in Figure 4D, the peak areas increased with the lower injection pressure, probably because the analytes may need to pass through the sorbent beads more slowly to allow enough time for a proper interaction between the analytes and the $C_{18}$ sorbent. In view of these results, a sample loading pressure of 100 mbar was selected in our study with the purpose to achieve low LODs Next, the sample injection time was studied between 10 and 40 min by applying 100 mbar, using a 500 ng/mL standard solution of the four analytes, since injecting large volumes of samples represents the simplest way to increase sensitivity and therefore obtain lower LODs. The experimental results showed that for COC, COD, and MOR, the peak area increased with the sample loading time up to 30 min whereas, over this time, the peak areas remained relatively constant. In the case of MTD, the peak area increased with the sample loading time up to 40 min, meaning that the breakthrough volume of the $C_{18}$ sorbent for this analyte was not exceeded. In the end, to avoid prolonging the overall analysis time excessively, it was decided that a sample loading time of 30 min was reasonable, so this time was chosen to validate the analytical method.

Figure 5 shows the electro-pherogram obtained under the optimal in-line MSPE-CE conditions when a standard solution at pH 9.0 containing 100 ng/mL of COC, COD, and MTD, and 200 ng/mL of MOR was loaded at 100 mbar for 30 min.

### 3.3. Evaluation of the in-line MSPE-CE

The proposed in-line MSPE-CE procedure was validated in terms of linearity, repeatability, reproducibility, LOD, LOQ, percentage recovery, and enrichment factor using standard solutions.

In order to evaluate the linearity, six standard solutions containing the target compounds at concentrations between the LOQ and 500 ng/mL were prepared and injected into the in-line MSPE-CE system in triplicate. Calibration curves were constructed by plotting peak areas as a function of

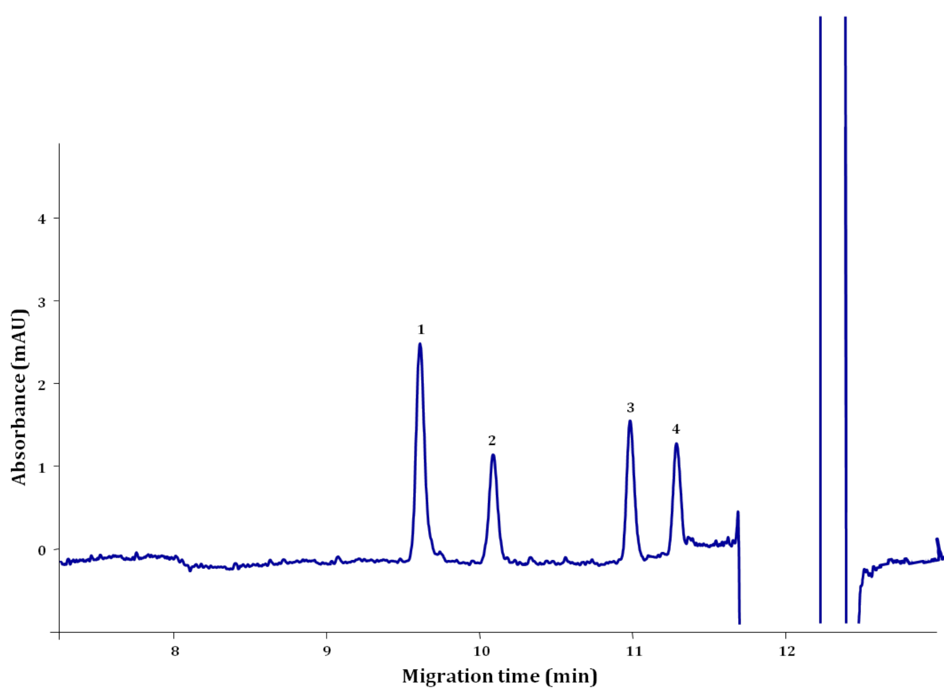


**Figure 5.** Electropherogram obtained under the optimal in-line MSPE-CE conditions when a standard solution at pH 9.0 containing 100 ng/mL of COC, COD and MTD, and 200 ng/mL of MOR was loaded at 100 mbar for 30 min. Other experimental conditions are reported in the text. Peak assignments: 1, MTD; 2, COC; 3, COD and 4, MOR.

the concentration and are summarized in Table 2. As can be seen in this table, good linearity was obtained with regression coefficients ($r^2$) greater than 0.998.

The precision was examined at three different concentrations. Repeatability was evaluated by injecting three replicates of each concentration level in duplicate on the same day and under identical experimental conditions. Intermediate precision was assessed on five consecutive days by injecting three replicates of each concentration level each day. The results presented in Table 2 are expressed as the %RSD of the peak areas and migration times.

The LODs were determined as the concentrations corresponding to three times the noise signal ($n$=5) and the LOQs as the lowest calibration level of each compound. The corresponding values are shown in Table 2.

In order to assess the recovery percentages and enrichment factors of the proposed in-line MSPE-CE

**Table 2.** Analytical performance parameters for the in-line magnetic SPE-CE-DAD.

| | *COC* | *COD* | *MTD* | *MOR* |
|---|---|---|---|---|
| **Linearity (ng/mL)** | 10–100 | 6–100 | 3–100 | 50–500 |
| **Calibration curve** [a] | y=0.0096x +0.1545 | y=0.0065x +0.5049 | y=0.0241x +1.0095 | y=0.0032x +0.7374 |
| **$r^2$** | 0.9992 | 0.9980 | 0.9982 | 0.9986 |
| **LOD (ng/mL)** | 5 | 2 | 0.5 | 20 |
| **Intra-day RSD of peak area (%, n=6)** | | | | |
| 20 ng/mL | 16.5 | 13.8 | 10.2 | - |
| 50 ng/mL | 9.4 | 4.8 | 4.6 | 6.2 |
| 100 ng/mL | 6.6 | 6.5 | 5.5 | 7.1 |
| **Intra-day RSD of migration time (%, n=6)** | | | | |
| 20 ng/mL | 0.4 | 0.4 | 0.7 | - |
| 50 ng/mL | 0.5 | 0.5 | 0.6 | 0.5 |
| 100 ng/mL | 0.5 | 0.5 | 0.7 | 0.6 |
| **Inter-day RSD of peak area (%, n=15)** | | | | |
| 20 ng/mL | 17.1 | 13.0 | 11.7 | - |
| 50 ng/mL | 7.1 | 5.4 | 6.5 | 9.2 |
| 100 ng/mL | 9.0 | 7.8 | 5.5 | 7.7 |
| **Inter-day RSD of migration time (%, n=15)** | | | | |
| 20 ng/mL | 0.6 | 1.0 | 0.9 | - |
| 50 ng/mL | 1.2 | 1.4 | 0.6 | 1.5 |
| 100 ng/mL | 0.5 | 0.4 | 0.7 | 0.6 |

[a] y: peak area value (mAU x seconds); x: concentration (ng/mL)

procedure, the calibration curves and LODs (S/N=3) of the CE-DAD system without the in-line preconcentration step were determined. The recovery percentages were calculated as the ratio of slopes provided by the calibration curves with and without the preconcentration step. To do so, the calibration curves of both methods were generated by plotting peak areas as a function of the amount of analyte injected, which should be the same in both methods. In this context, the calibration range was 0.01 – 1.50 ng. Table 3 shows the calibration curves, the $r^2$ values and the recovery percentages obtained for all the analytes. Recovery percentages

**Table 3.** Analytical performance parameters for the CE with and without the in-line preconcentration step.

| | *COC* | *COD* | *MTD* | *MOR* |
|---|---|---|---|---|
| **CE WITHOUT THE IN-LINE PRECONCENTRATION STEP** | | | | |
| **Linearity (ng)** | 0.04–0.50 | 0.02–0.50 | 0.01–0.50 | 0.10–1.50 |
| **Calibration curve [a]** | y=7.7453x +0.8295 | y=3.5617x +0.1848 | y=7.7521x +0.8415 | y=3.5416x +0.1906 |
| **$r^2$** | 0.9988 | 0.9985 | 0.9995 | 0.9984 |
| **LOD (ng/mL)** | 1,500 | 900 | 350 | 2,500 |
| **CE WITH THE IN-LINE PRECONCENTRATION STEP** | | | | |
| **Linearity (ng)** | 0.04–0.50 | 0.02–0.50 | 0.01–0.50 | 0.10–1.50 |
| **Calibration curve [a]** | y=6.0810x +0.0965 | y=2.7602x +0.4132 | y=6.4815x +0.8315 | y=2.5603x +0.1000 |
| **$r^2$** | 0.9983 | 0.9981 | 0.9989 | 0.9974 |
| **Recovery percentages (%)** | 78.5 | 77.5 | 83.6 | 72.3 |
| **Enrichment factors** | 300 | 450 | 700 | 125 |

[a] y: peak area value (mAU x seconds); x: amount of analyte injected (ng)

of 78.5, 77.5, 83.6, and 72.3% in aqueous medium were calculated for COC, COD, MTD, and MOR, respectively.

The enrichment factors were calculated as the ratio of LODs obtained with and without the preconcentration step and are also listed in Table 3. As shown in this table, the established in-line MSPE-CE method allows improving the detection sensitivity up to 700-fold compared with CE without preconcentration.

All of the data presented in Tables 2 and 3 indicate that the developed method in this work shows suitable efficiency and sensitivity for determining illicit drug use in therapeutic drug monitoring, drug rehabilitation programs, and doping control, and even in forensic cases.

For example, concentrations of COD and MOR in the range of 59 ─160,600 ng/mL and 52 ─1,122,000 ng/mL, respectively, were found in the urine samples of 13,126 pain patients [30]. In a case of doping, urine concentrations of MOR and COD of 54,500 ng/mL and 52 700 ng/mL, respectively, were reported [31]. The concentrations encountered in blood in a post-mortem case were 743 ng/mL for MOR, 24 ng/mL for COD and 65 ng/mL for COC [32].

In addition, it should be highlighted that, compared with the CE- based methods that use the conventional in-line SPE set-ups for sample preparation and preconcentration of the analytes, the method developed here using MPs as solid-phase supports for in-line SPE proved to be greatly simplified since the MPs can easily be manipulated inside the CE capillary simply by using a pair of magnets. In this context, it can be concluded that the use of MPs is an easier and faster way to create a concentrator inside the CE capillary, which does not require complicated and time consuming procedures for fabrication. Moreover, the configuration proposed in this paper made it possible to replace the magnetic adsorbents by simply refilling the capillary with a fresh plug of MPs without needing taking the capillary with the concentrator out of the cassette, as is required when conventional in-line SPE configurations are used.

### 3.4. Figures of merit for urine samples

Drug-free pooled urine sample prepared by mixing urine collected from various non-addicted volunteers was spiked with the standards. Before being analyzed under the optimal in-line MSPE-CE conditions, the urine samples were subjected to an LLE procedure described in Section 2.4, which does not involve any preconcentration of the analytes. Figure 6 shows the electropherograms obtained of a blank urine sample (A) and a blank urine sample spiked with the investigated compounds at a concentration of 80 ng/mL(B). From the figure, it can be seen that no matrix peaks were found comigrating with the analytes and clean electropherograms with little chemical background noise were obtained.

The LLE/in-line MSPE-CE method was validated using pooled blank urine sample spiked with the analytes and the obtained results are summarized in Table 4. Calibration curves (six points, each calibration point was determined in triplicate), constructed using the matrix-matched calibration, gave a high level of correlation between peak areas and

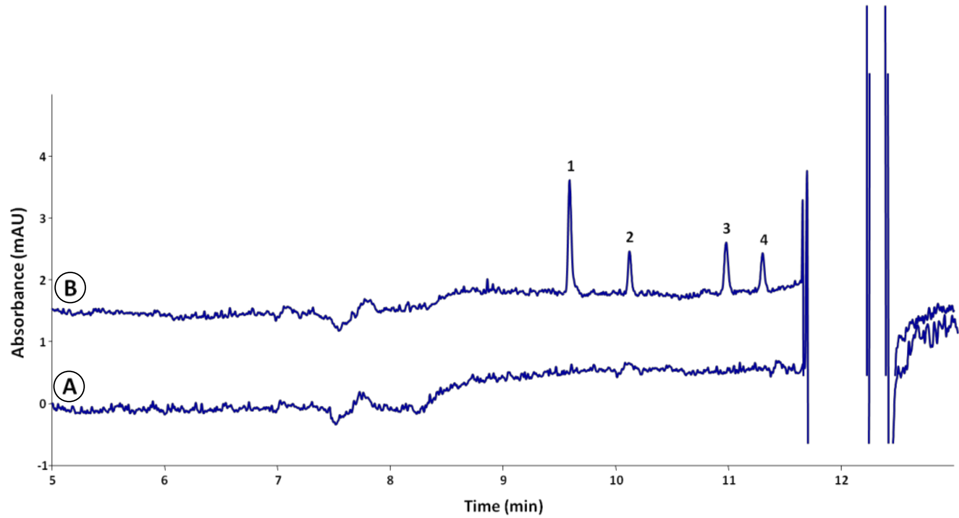


**Figure 6.** Electropherograms obtained under the developed LLE/in-line MSPE-CE method of a blank urine sample (A) and a blank urine sample spiked with the investigated compounds at a concentration of 80 ng/mL (B). Experimental conditions are reported in the text. Peak assignments: 1, MTD; 2, COC; 3, COD and 4, MOR.

concentrations with $r^2$ higher than 0.996. The calibration range should be selected with respect to the concentration levels expected for a certain application [33]. In the case of abusers, a wide variety of concentrations of the drugs may be found in their urine samples and, thus, a wider calibration range was selected to use with the aim to quantify in urine without further dilution.

LODs (S/N=3) of 50 ng/mL and LOQs (lowest point of the calibration curves) of 75 ng/mL were reached (Table 4), which are far below the cut-off concentrations of COC and opiates in urine samples (300 ng/mL [34]), making the developed method suitable for routine determination of illicit drugs in biological samples. The sensitivity achieved is comparable to the levels published in the literature [35–37]. For instance, Tang *et al.* [35] reported a LOD range of 1–50 ng/mL for COC, COD, MTD, and MOR in urine samples with SPE/ultra-HPLC (UHPLC)-MS/MS, while the assay by Hegstad *et al.* [36] found LODs in the range of 6 – 25 ng/mL for COD, MTD, and MOR in urine samples with UHPLC-MS/MS. However, it should be pointed out that, in our work, an inexpensive determination method using non-sophisticated instrumentation has successfully been developed.

**Table 4.** Regression equations, repeatability and reproducibility values, LODs and relative recoveries obtained for urine samples by LLE/in-line magnetic SPE-CE-DAD.

| | *COC* | *COD* | *MTD* | *MOR* |
|---|---|---|---|---|
| **Linearity (ng/mL)** | 75–5,000 | 75–5,000 | 50–5,000 | 75–5,000 |
| **Calibration curve** [a] | y=0.0134x -0.7452 | y=0.0182x -0.9712 | y=0.0204x +2.2049 | y=0.0172x -1.8509 |
| **$r^2$** | 0.9982 | 0.9991 | 0.9964 | 0.9981 |
| **LOD (ng/mL)** | 50 | 50 | 20 | 50 |
| **Intra-day RSD of peak area (%, n=6)** | | | | |
| 100 ng/mL | 14.3 | 15.1 | 15.8 | 14.8 |
| 500 ng/mL | 13.6 | 11.6 | 13.7 | 14.1 |
| 1,000 ng/mL | 11.8 | 12.1 | 12.5 | 11.2 |
| **Inter-day RSD of peak area (%, n=15)** | | | | |
| 100 ng/mL | 17.9 | 16.1 | 19.6 | 16.6 |
| 500 ng/mL | 16.1 | 17.6 | 16.2 | 15.6 |
| 1,000 ng/mL | 15.9 | 18.9 | 17.7 | 17.8 |
| **Relative recoveries (%, n=3)** | | | | |
| 100 ng/mL | 86.4 | 87.4 | 94.3 | 76.8 |
| 500 ng/mL | 85.0 | 81.9 | 90.5 | 75.9 |
| 1,000 ng/mL | 87.1 | 84.2 | 91.3 | 78.3 |

[a] y: peak area value (mAU x seconds); x: concentration (ng/mL)

As shown in Table 4, RSDs of peak areas lower than 20% for low, medium, and high concentrations, were obtained in both intra- and inter-day precision experiments.

The recovery study was carried out by comparing the peak areas corresponding to blank urine samples spiked with three different concentrations, with the peak area obtained for standard solutions containing the same concentration levels, all analyzed by using the proposed LLE/in-line MSPE-CE method. The mean extraction yields values were calculated from three independent analyses. Relative recoveries greater than 75.9% were obtained (Table 4). As can be seen, the proposed method provides satisfactory results in terms of recoveries and precision, so method

accuracy for drugs of abuse determination in urine samples was successfully demonstrated.

The data presented in Tables 2 and 4 show that the matrix effect is present and, thus, quantification of the urine samples from drug abusers was conducted with the matrix-matched calibration curves, found in Table 4, in order to ensure reliable and accurate results.

### 3.5. Urine samples from drug abusers

The validated LLE/in-line MSPE-CE method was applied to four urine samples collected from four patients undergoing MTD maintenance treatment. The electropherogram of a patient's urine is shown in Figure 7. The identification of the drugs of abuse was performed considering their migration times and absorption spectra. To confirm the presence of each analyte, a standard addition was made to each sample extract. An increase in the peak area confirmed the presence of the illicit substances. Three replicates of each urine sample were performed by repetitive application of the whole procedure.

The four urine samples were positive for MTD with concentrations of 812.6–4337.6 ng/mL, because it was the prescribed drug for the four patients. As is well-known, MTD is used therapeutically in maintenance treatment of heroin addicts. In this context, the results obtained are coherent. COC was found in one of the cases with a concentration of 324.9 ng/mL. This finding is in accordance with the data provided by the individual at the time of sampling. The RSD was less than 20% in all analyses. COD and MOR were not detected in any of the four samples.

However, the results obtained are not enough to prove the abstinence and compliance to the therapy of the four individuals because, as is well-known, most drugs of abuse are metabolized once ingested. As such, the metabolites of COC and MTD should be considered as well. In this context, further work will be necessary to determine the possible presence of those metabolites in order to confirm the compliance to the therapy of the drug abusers.

## 4. Concluding remarks

In this research, a new SPE procedure coupled in-line to CE using functionalized MPs as adsorbents has been successfully developed for the analysis of drugs of abuse. This method provides an interesting approach in terms of carrying out sample preparation and preconcentration of the target compounds inside the capillary without the need to use

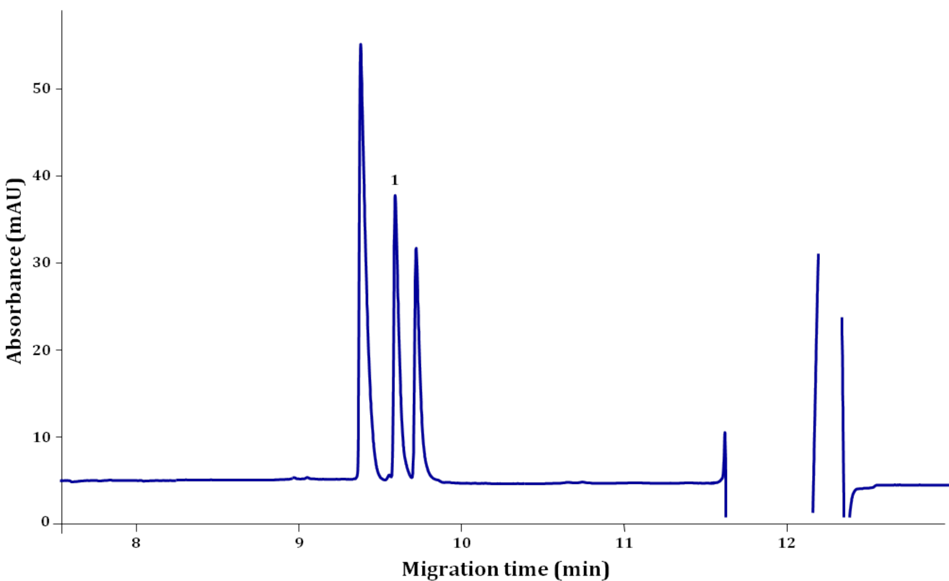


**Figure 7.** Electropherogram of a patient's urine obtained under the developed LLE/in-line MSPE-CE method. Peak assignments: 1, MTD.

the conventional in-line SPE-CE configurations that have to be constructed by the user. It therefore offers an easier and faster way to create a concentrator inside the capillary with the possibility of renewing the magnetic adsorbents simply by refilling the capillary with a fresh plug of MPs. The developed in-line MSPE-CE method has proven to be suitable for determining drugs of abuse in urine samples from drug abusers, making it a very useful diagnostic tool for routine determination of illicit drug use in therapeutic drug monitoring, drug rehabilitation programs and doping control. Further work could focus on exploiting this novel strategy in the determination of drugs of abuse and their metabolites from other different complex biological samples, such as hair.

**Acknowledgment**

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## 3.2.2. Discussion of results

This section outlines the most important aspects of our research work focused on using functionalized magnetic particles as the adsorbent for the construction of an in-line SPE analyte concentrator in CE to be applied in the analysis of DOAs in urine.

In short, the in-line magnetic SPE-CE procedure proposed here for determining DOAs (Figure 2) involves firstly pumping the magnetic particles into the capillary, where they congregate between the two magnets and form a plug (step 1). Then, the sample is injected at a low pressure into the capillary and through the plug, causing the drug molecules to bind to the $C_{18}$ coating (step 2). After the sample clean-up with electrolyte solution (step 3), methanol containing formic acid is pumped through the capillary to release the drug molecules from the magnetic particles (steps 4 and 5), after which these are separated by CE and detected by UV detection (step 6). Following the analysis, the magnetic particles are flushed out of the capillary at a high pressure, making the capillary ready for another round (step 7).

The relevance of this methodology lies in the excellent advances that have been achieved in the fabrication process of the analyte concentrator. The in-line SPE-CE configuration developed enabled: (i) the synthesized magnetic particles to be directly loaded into the CE capillary simply by using an aqueous suspension of the particles; and, (ii) the use of a very high pressure (12,000 mbar) to remove the SPE sorbents from the capillary without needing to take the magnets out of the cassette. These two facts significantly simplify the procedure of loading and unloading the SPE sorbent compared to conventional in-line SPE-CE configurations, allowing the particles to be replaced very easily and quickly after each analysis, which can be advantageous for preventing possible cross-contamination and/or carryover. Furthermore, it is also worth mentioning, that the possibility of holding the magnetic particles in their place during sample loading and rinsing steps avoids the use of frits and their associated problems (see Section 1.4.).

Furthermore, the established method proved suitable for increasing the detection sensitivity up to 700 times in comparison to CE without preconcentration, enabling the analytes of interest to be detected in urine samples at concentrations of 20-50 ng/mL, by using magnetic sorbents prepared in-house and located inside a 50-μm i.d. capillary simply equipped with two magnets that are commercially available and therefore accessible to everyone. The use of instrumentation that is very simple and easy to handle makes our approach a reasonably attractive tool for routine analysis

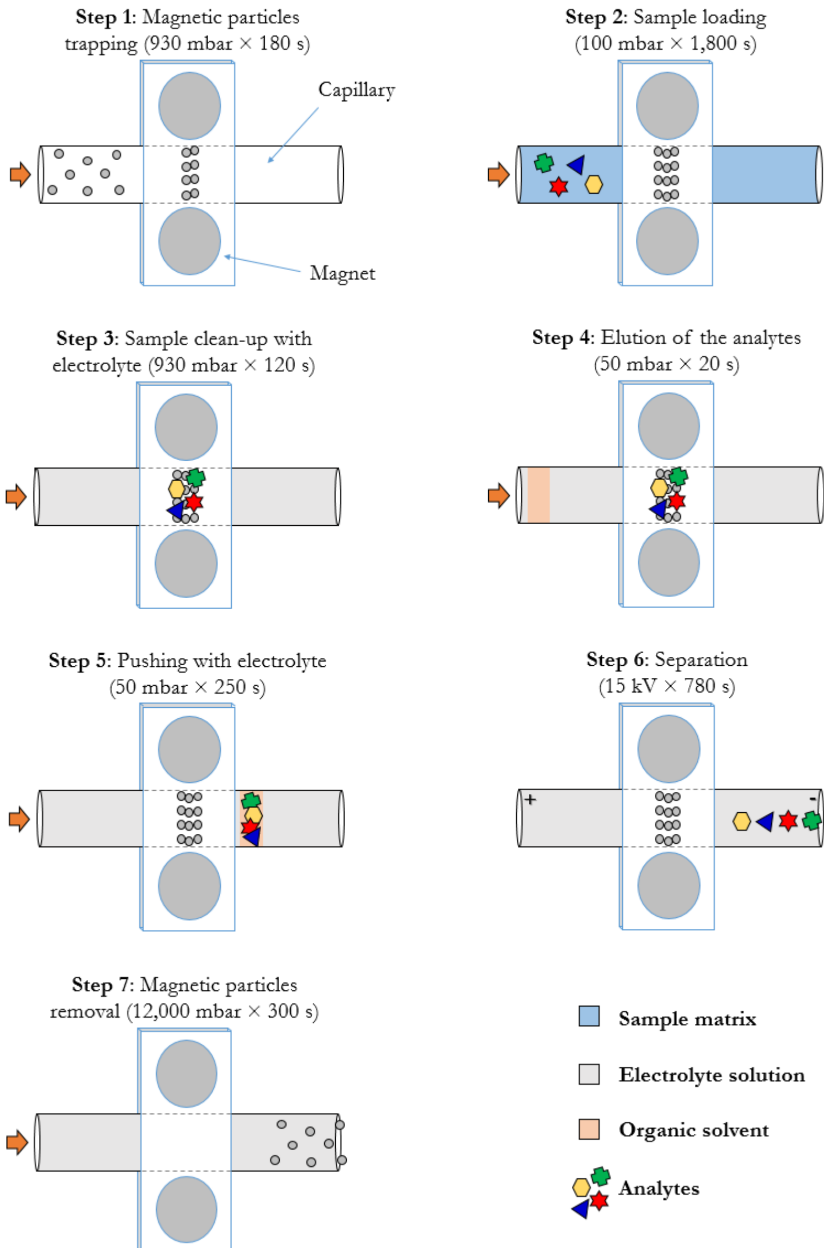


**Figure 2.** Schematic representation of the different steps involved in the in-line magnetic SPE-CE developed.

of DOAs. In addition, the LODs obtained here are below the cut-off levels for these compounds in urine {about 300 ng/mL [1-3]}. Therefore, the sensitivity achieved using a simple UV detector is suitable for determining drug (ab)use in therapeutic drug monitoring, drug rehabilitation programs, doping control, workplace and even in forensic cases.

Combining magnetic particles in-line with CE is not a common approach for the determination of small molecules. Actually, to the extent of our knowledge, this study is the second one to date in the literature dealing with such a topic and the first focuses on presenting an application for biological samples. Urine samples obtained from four patients undergoing treatment with methadone to wean them off heroin were satisfactorily analysed using the developed method with a simple pretreatment procedure based on LLE. As expected, methadone was found in the urine from all the patients. In addition, cocaine was detected in the urine from one of the patients, who admitted using it.

The promising results achieved here encourage further work in this field. Future research could focus on exploiting this novel strategy in the determination of DOAs and their metabolites in hair samples to obtain complementary information of an individual's drug (ab)use pattern, since, as already discussed, DOAs can be detected in urine for no more than 2-3 days. Future work also can be aimed at investigating other sorbent materials with different chemical properties from $C_{18}$ to provide different selectivity and, moreover, the combination between magnetic SPE and electrophoretic preconcentration techniques, such as t-ITP, to attempt to decrease the LODs further.

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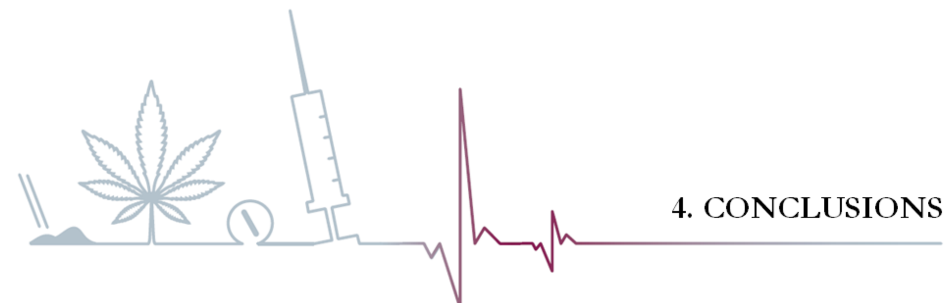

# 4. CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES
Tatiana Baciu

The most important conclusions derived from the studies presented in the present Doctoral Thesis can be summarized as follows:

- Four approaches for drug testing in biological samples using simple-to-handle instrumentation were successfully developed.

- The in-line SPE-CE strategies proved to be highly useful for improving the sensitivity of CE in the analysis of recreational DOAs belonging to different classes (stimulants, narcotics and new psychoactive substances) in hair and urine samples.

- The developed methodologies for testing DOAs and metabolites in biological samples exhibit suitable sensitivity for determining illicit drug (ab)use in therapeutic drug monitoring, drug rehabilitation programs, doping control, workplace and even forensic cases.

- CE using CDs as electrolyte additives, namely α-CD and β-CD, was successfully applied for the enantioselective separation of the chiral drugs investigated. In addition, α-CD proved to be highly useful for improving the resolution of closely related non-chiral compounds, such as cocaine and opiates.

- PLE was demonstrated to be a useful technique for extracting the compounds selected from hair samples in a very short period of time compared to acidic digestions, commonly used for such a task. The established PLE procedures proved to be green, especially when compared to procedures involving the use of organic solvents.

- The hair extracts obtained after the PLE process could be directly injected into the in-line SPE-CE systems without needing any evaporation and reconstitution steps, thereby considerably reducing the sample preparation time.

- Segmental hair analysis by means of in-line SPE-CE-UV proved to be a suitable diagnostic tool for determining drug (ab)use histories, providing DOAs concentrations in different periods of time, depending on the length of

hair sample, being useful in forensic toxicological laboratories, as well as rehabilitation and addiction treatment programs.

- The findings in the hair samples of four drug abusers undergoing a rehabilitation program were generally in line with the attempted detoxification and the data provided by them at the time of sampling.

- The developed method using magnetic particles as solid-phase supports for in-line SPE-CE proved to be greatly simplified, since the magnetic particles can be easily manipulated inside the CE capillary simply by using a pair of magnets. In this way, using magnetic particles proved to be an easy and quick way to create an analyte concentrator inside a 50 μm i.d. capillary, which does not require complicated and time-consuming procedures for fabrication.

- The in-line magnetic SPE-CE configuration proposed enabled the removal of the magnetic particles from the capillary by simply applying a high pressure without needing to remove the capillary with the concentrator from the cassette, as is required when using the traditional in-line SPE-CE configuration. In addition, replacing the magnetic particles was achieved by simply injecting a fresh plug of the particles into the CE capillary.

- Using silica-coated $Fe_3O_4$ particles functionalized with $C_{18}$ groups coupled in-line with CE proved to be an excellent analytical tool for rapid testing of DOAs in urine samples, being very useful in laboratories where hundreds of samples are analysed every day.

- In all our studies, a green chemistry approach was taken into consideration, always striving to minimize the volume of organic solvents used. Therefore, the in-line SPE-CE procedures established can be considered highly environmentally friendly due to the very low quantity of organic solvent consumed (20-24 nL).

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# APPENDIX

UNIVERSITAT ROVIRA I VIRGILI
SOLID-PHASE EXTRACTION COUPLED IN-LINE TO CAPILLARY ELECTROPHORESIS:
DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES
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**Appendix I.** Abbreviations used in this Doctoral Thesis.

| | |
|---|---|
| $[Ru(bpy)_3]Cl_2$ | Tris(bipyridine)ruthenium (II) chloride |
| 2-4-MPEA | 2-(4-Methoxyphenyl)ethylamine |
| 2D GC | Two-dimensional gas chromatography |
| 2-MeOPP | 1-(2-Methoxyphenyl)piperazine |
| 2-MMA | 2-methoxy-*N*-methylaniline |
| 3-AM | 3-Acetylmorphine |
| 4-FA | 4-Fluoroamphetamine |
| 4-MEC | 4-Methylethcathinone |
| 6-AC | 6-Acetylcodeine |
| 6-AM | 6-Acetylmorphine |
| 7-AC | 7-Aminoclonazepam |
| 7-AF | 7-Aminoflunitrazepam |
| 7-AN | 7-Aminonitrazepam |
| 18-C-6-TA | 18-crown-6-tetracarboxylic acid |
| α-lac | Alfa-lactalbumin |
| α-PVP | Alfa-pyrrolidinovalerophenone |
| β-HMP | Beta-hydroxy-methamphetamine |
| β-lg | Beta-lactoglobulin |
| AA | Acetic acid |
| ACN | Acetonitrile |
| AGP | Alpha-1 acid glycoprotein |
| AM-C-BD | 5-aminomethyl-7-chloro-1,3-benzodioxole |
| AP | Amphetamine |
| APCI | Atmospheric pressure chemical ionization |
| APs | Amphetamines |
| ASEI | Anion selective exhaustive injection |
| ATS | Amphetamine-type stimulants |
| BGE | Background electrolyte |
| BSTFA | *N*,*O*-bis(trimethylsilyl)tifluoroacetamide |
| BZDs | Benzodiazepines |
| BZE | Benzoylecgonine |
| $C^4D$ | Capacitively coupled contactless conductivity detector |
| C6G | Codeine-6-glucuronide |
| $C_{18}$ | Octadecyl |
| CD | Cyclodextrin |

| | |
|---|---|
| CE | Capillary electrophoresis |
| CET | Cocaethylene |
| CM-β-CD | Carboxymethyl-beta-cyclodextrin |
| $CO_2$ | Carbon dioxide |
| COC | Cocaine |
| COD | Codeine |
| CSEI | Cation-selective exhaustive injection |
| CZE | Capillary zone electrophoresis |
| DAD | Diode array detector |
| DAPPI | Desorption atmospheric pressure photoionization |
| DART | Direct analysis in real-time mass spectrometry |
| DCM | Dichloromethane |
| DexAP | Dextroamphetamine |
| DESI | Desorption electrospray ionization |
| DHCOD | Dihydrocodeine |
| DHNK | 5,6-Dehydronorketamine |
| DLLME | Dispersive liquid-liquid microextraction |
| DMA | Dimethylamphetamine |
| DMD | Desmethyldiazepam |
| DMMA | Dimethylamylamine |
| DOAs | Drugs of abuse |
| DOET | 2,5-Dimethoxy-4-ethylamphetamine |
| DOM | 2,5-Dimethoxy-4-methylamphetamine |
| DOPR | 2,5-Dimethoxy-4-propylamphetamine |
| DynA | Dynorphin A |
| DZP | Diazepam |
| EC/ECL | Electrochemical detector |
| EDDP | 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine |
| EI | Electron impact |
| EME | Ecgonine methyl ester |
| End1 | Endomorphin-1 |
| End2 | Endomorphin-2 |
| EP | Ephedrine |
| ESI | Electrospray ionization |
| EtAc | Ethyl acetate |
| f.t. | Film thickness |
| FA | Formic acid |

| | |
|---|---|
| FASI | Field-amplified sample injection |
| $Fe_3O_4$ | Magnetite (iron oxide) |
| GC | Gas chromatography |
| GHB | Gamma-hydroxybutyric acid |
| GHB-Gluc | GHB-β-*O*-glucuronide |
| GHB-Sulf | GHB-4-sulfate |
| IT | Ion trap |
| LC | Liquid chromatography |
| LIF | Laser-induced fluorescence detector |
| LLE | Liquid-liquid extraction |
| LLOQ | Low limit of quantification |
| LOD | Limit of detection |
| LODs | Limits of detection |
| LOQ | Limit of quantification |
| LSD | Lysergic acid diethylamide |
| LVSEI | Large volume sample injection |
| KET | Ketamine |
| HCl | Hydrochloric acid |
| HFBA | Heptafluorobutyric acid |
| HFIP(OH) | Hexafluoroisopropanol |
| HF-LPME | Hollow fiber liquid-phase microextraction |
| HILIC | Hydrophilic interaction liquid chromatography |
| HLB | Hydrophilic-lipophilic balance |
| HNK | Hydroxynorketamine |
| HP-β-CD | 2-Hydroxypropyl-beta-cyclodextrin |
| HPLC | High performance liquid chromatography |
| HRMS | High resolution mass spectrometry |
| HS-γ-CD | Highly sulfated-gamma-cyclodextrin |
| HS-SPME | Head space solid-phase microextraction |
| HYCOD | Hydrocodone |
| HYMOR | Hydromorphone |
| IA | Immunoaffinity |
| i.d. | Internal diameter |
| IgG | Immunoglobulin G |
| IUPAC | International union of pure and applied chemistry |
| M3G | Morphine-3-glucuronide |
| M6G | Morphine-6-glucuronide |

| | |
|---|---|
| MAE | Microwave-assisted extraction |
| MALDI | Matrix-assisted laser desorption/ionization |
| MBA | 2-bromo-*N*-methylbenzylamine |
| MBDB | Benzodioxolyl-*N*-methylbutanamine |
| MBTFA | *N*-methyl-bis(trifluoroacetamide) |
| MCAT | Methcathinone |
| MCX | Mixed-mode cation exchange |
| mCPP | Meta-chlorophenylpiperazine |
| MDA | 3,4-Methylenedioxyamphetamine |
| MDEA | 3,4-Methylenedioxyethylamphetamine |
| MDMA | 3,4-Methylenedioxymethamphetamine |
| MDPV | 3,4-Methylenedioxypyrovalerone |
| MEKC | Micellar electrokinetic chromatography |
| MEEKC | Microemulsion electrokinetic chromatography |
| MeOH | Methanol |
| MEPH | Methylephedrine |
| MEPS | Microextraction by packed sorbent |
| Met | Methionine-enkephaline |
| MIP | Molecularly imprinted polymer |
| MISPE | Molecularly imprinted solid-phase extraction |
| MOR | Morphine |
| mM | mmol/L |
| MMBA | 3-Methyl-*N*-methylbenzylamine |
| MP | Methamphetamine |
| MPEA | 2-Methoxyphenethylamine |
| MPH | Methylphenidate |
| MRM | Multiple reaction monitoring |
| MS/MS | Tandem mass spectrometry |
| MS | Mass spectrometry |
| MSPE | Magnetic solid-phase extraction |
| MSS | Micelle to solvent stacking |
| MSTFA | *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide |
| MTD | Methadone |
| NaOH | Sodium hydroxide |
| n.d. | Not determined |
| n.q. | Not quantified |
| NE | Norephedrine |

| | |
|---|---|
| NKET | Norketamine |
| NPE | Norpseudoephedrine |
| NPS | New psychoactive substances |
| NTA | Nitrilotriacetic acid |
| o-MWNTS | Oxide multi-walled carbon-nanotubes |
| o.d. | Outer diameter |
| OXCOD | Oxycodone |
| OXMOR | Oxymorphone |
| PCA | Para-chloroamphetamine |
| PCP | Phencyclidine |
| PCX | Polymeric mixed-mode cation exchange |
| PE | Pseudoephedrine |
| PEA | 2-Phenethylamine |
| PFBCI | Pentafluorobenzoyl chloride |
| PFP | Pentafluorophenyl |
| PFPA | Perfluoropropionic anhydride |
| PLE | Pressurized liquid extraction |
| PMA | Paramethoxyampehtamine |
| PMMA | Paramethoxymethamphetamine |
| PMMA* | Polymethyl methacrylate |
| PTFE | Polytetrafluoroethylene |
| Pyr | Pyridine |
| QqQ | Triple quadrupole |
| QTrap | Quadrupole ion trap |
| QTOF | Quadrupole time-of-flight |
| QuEChERs | Quick, easy, cheap, effective, rugged and fast |
| RPLC | Reversed-phase liquid chromatography |
| RSDs | Relative standard deviations |
| S-γ-CD | Sulfated-gamma-cyclodextrin |
| SALDI | Surface assisted laser desorption/ionization |
| SALLE | Salt assisted liquid-liquid extraction |
| SCX | Strong-cation exchange |
| SDS | Sodium dodecyl sulphate |
| SDME | Single drop microextraction |
| SFC | Supercritical fluid chromatography |
| SLE | Solid supported liquid-liquid extraction |
| SPE | Solid-phase extraction |

| | |
|---|---|
| SPME | Solid-phase microextraction |
| TCA | Trichloroacetic acid |
| TEA | Triethylamine |
| THC | $\Delta^{9}$-Tetrahydrocannabinol |
| THCA | $\Delta^{9}$-Tetrahydrocannabinol-9-carboxylic acid |
| THC-COOH | 11-Nor-9-carboxy-$\Delta^{9}$-tetrahydrocannabinol |
| THC-OH | 11-Hydroxy- $\Delta^{9}$-Tetrahydrocannabinol |
| TFA | Trifluoroacetic acid |
| TFMPP | 3-Trifluoromethylphenylpiperazine |
| t-ITP | Transient isotachophoresis |
| TMAH | Tetramethylammonium hydroxide |
| TMCS | Trimethylchlorosilane |
| TMS | Trimethylsilyl |
| TMSH | Trimethylsulfonium hydroxide |
| TOF | Time-of-flight |
| TTR | Transthyretin |
| UA-LDS | Ultrasound-assisted low density solvent |
| UHPLC | Ultra-high performance liquid chromatography |
| UHPSFC | Ultra-high performance supercritical fluid chromatography |
| UNODC | United Nations Office on Drugs and Crime |
| USAEME | Ultrasound-assisted emulsification-microextraction |
| UV | Ultraviolet detection |
| v/v | Volume/volume |
| w/v | Weight/volume |
| WADA | World anti-doping agency |

**Appendix II.** Name, structure and $pK_a$ values of the analytes investigated in the present Doctoral Thesis.

| *Name* | *Structure* | *$pK_a^{(*)}$* |
|---|---|---|
| 6-Acetylmorphine | | 8.03<br>9.41 |
| Benzoylecgonine | | 3.35<br>10.83 |
| Cocaine | | 8.97 |
| Codeine | | 8.23<br>13.40 |

| *Name* | *Structure* | $pK_a$(*) |
|---|---|---|
| Methadone | | 9.05 |
| Morphine | | 8.25<br>9.48 |
| *R*,*S*-4-methylephedrine | | n.d. |
| *R*,*S*-3,4-methylenedioxypyrovalerone | | 8.41 |
| *R*,*S*-mephedrone | | 7.41 |

(*) SciFinder, Chemical Abstracts Service, pKa, Columbus, OH, 2017, https://origin-scifinder.cas.org (accessed 21.05.17); Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2017 ACD/Labs).

**Appendix III.** List of publications.

Tatiana Baciu, Francesc Borrull, Carme Aguilar, Marta Calull, *Recent trends in analytical methods and separation techniques for drugs of abuse in hair*, Analytica Chimica Acta 856 (2015) 1-26.

Tatiana Baciu, Igor Botello, Francesc Borrull, Marta Calull, Carme Aguilar, *Capillary electrophoresis and related techniques in the determination of drugs of abuse and their metabolites*, Trends in Analytical Chemistry 74 (2015) 89-108.

Tatiana Baciu, Francesc Borrull, Marta Calull, Carme Aguilar, *Determination of cocaine in abuser hairs by CE: monitoring compliance to a detoxification program*, Bioanalysis 7 (2015) 437-447.

Tatiana Baciu, Francesc Borrull, Carme Aguilar, Marta Calull, *Findings in the hair of drug abusers using pressurized liquid extraction and solid-phase extraction coupled in-line with capillary electrophoresis*, Journal of Pharmaceutical and Biomedical Analysis 131 (2016) 420-428.

Tatiana Baciu, Francesc Borrull, Marta Calull, Carme Aguilar, *Enantioselective determination of cathinone derivatives in human hair by capillary electrophoresis combined in-line with solid-phase extraction*, Electrophoresis 37 (2016) 2352-2362.
(Special Issue: Focus on Bioseparations)

Tatiana Baciu, Francesc Borrull, Christian Neusüß, Carme Aguilar, Marta Calull, *Capillary electrophoresis combined in-line with solid-phase extraction using magnetic particles as new adsorbents for the determination of drugs of abuse in human urine*, Electrophoresis 37 (2016) 1232-1244.
(Special Issue: Concentration and Sensitivity Enhancement)

Tatiana Baciu, Francesc Borrull, Carme Aguilar, Marta Calull, *Sensitivity enhancement in capillary electrophoresis using magnetic particles as solid-phase extraction sorbents for the determination of drugs of abuse in urine* in: R.A. Musah (Ed.) Analysis of drugs abuse: methods and protocols (Methods in Molecular Biology), Springer New York, 2017.
(Book chapter submitted)