Role of LC-MS/MS in hair testing for the determination of common drugs of abuse and other psychoactive drugs

Abstract

Hair testing has been used in toxicological investigations for the last two decades, but only recently a remarkable extension of hair analysis to a variety of application fields was observed, besides drug abuse recognition. The dramatic improvements of LC–MS/MS instrumentation make the detection of tiny amounts of almost whatever drug in hair possible, even after single-dose intake. The progresses observed during the last 5 years in the detection of psychoactive substances in hair are reviewed herein. The literature is partitioned according to the target compounds, namely traditional drugs of abuse, new psychoactive substances and pharmaceutical psychoactive substances. The LC–MS/MS methods presented are addressed to determine a single class of drugs, with the primary aim of accurate quantitation, or to perform multiclass analysis, for rapid and effective screening protocols.

Introduction

Hair analysis currently represents a reliable and well-established means of clinical and forensic investigations. The international community (in particular, the Society of Hair Testing) periodically meets to exchange scientific experiences, establish new protocols and consensus documents, in order to provide more thorough interpretation of analytical results and consequent clinical or legal judgments. As a matter of fact, hair (segmental) analysis is regularly requested in clinical and forensic investigations to evaluate drug exposure [1-5], to portray drug abuse history and/or withdrawal control, to perform workplace drug testing [6], driving re-licensing and post-mortem investigations, to ascertain drug-facilitated crimes, and occasional or pre-natal exposure to drugs [7]. In particular, hair is the most helpful specimen when (i) long-time retrospective information on drug consumption is of interest; (ii) differentiation between single exposure and habitual drug abuse has to be established; (iii) urine and blood specimens cannot be timely collected, because of the delay in reporting a drug-facilitated crime, or because biological fluids may not be available anymore, as in some post-mortem investigations.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is gradually replacing GC-MS in both screening and confirmation procedures and is increasingly acknowledged as the technique of choice for hair analysis. This trend depends on the increasing performances and decreasing costs of LC-MS/MS instrumentation, the absence of derivatization steps, and the multi-target capability of LC-MS/MS protocols. Drug testing in hair samples widely took profit from technology improvements of both chromatographic and spectrometric counterparts, which include the introduction of ultra-high-performance LC, the progressive refinement of triple quadrupole design, the wide recognition of TOF and hybrid mass analyzers, and the achievements of fast electronics. These elements altogether produced potent impact on the analytical potential toward the inquiries of toxicological interest, particularly those concerning hair samples. Widely improved sensitivity, extended coverage of analytes, and high throughput laboratory activity represent recent

accomplishment in toxicological hair analysis, owed to modern LC-MS/MS instrumentation and methods. The literature of the last five years on the detection of psychoactive substances by LC-MS/MS is reviewed herein.

Drugs of abuse

In order to meet the high demand for drug testing in hair specimens, several novel analytical methods have been developed, validated and published in the last years, most of which were addressed to class-specific groups of compounds or multi-class drugs.

Class-specific drug methods: amphetamines

Amphetamines (APs) and methamphetamines (MAs) are generally ranked among the traditional drugs of abuse, because they have been abused since the early decades of the last century as potent central nervous system (CNS) stimulants. However, several new AP and MA derivatives have been subsequently synthesized, including 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethamphetamine (MDEA), and N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), in order to magnify and expand their psychoactive effects, making the illegal use of this class of substances, particularly among adolescents, a still growing social problem worldwide. Therefore, in the last five years, several new LC-based methods were developed to improve the detection of AP and MA analogues in hair analysis. The main advantage is given from the combination of improved sensitivity of the LC-MS/MS determinations together with a generally fast sample preparation, especially if compared to GC-MS analyses where a derivatization step is always required [8-10]. The need of superior sensitivity provided by LC-MS/MS turned out crucial in the detection of single exposure to MDMA [10].

A dedicated LC-MS/MS protocol was developed with the scope of monitoring the incorporation of AP and MA in hair after controlled MA administration [8]. From 10 mg of decontaminated hair, MA and AP concentrations, respectively in the intervals 0.60-3.53 ng/mg and 0.13-0.30 ng/mg, were found after ingestion of four 10-mg doses of S-(+)-methamphetamine hydrochloride, within one week. Moreover, significant dependence (*i.e.*, direct linear correlation) of the degree of drug **incorporation into hair** and melanin content was clearly demonstrated. A similar LC-MS/MS approach for the determination of MA and AP in micropulverized hair, using only 2 mg of sample, was previously published by Miyaguchi *et al.* [9]. The method used a fast micropulverized extraction in acidic water/acetonitrile solution in comparison with two conventional methods (acidic-methanolic extraction and alkaline digestion) and was tested on five real samples from drug abusers, showing no statistically significant differences in AP and MA detected concentrations among the three procedures.

Other analytical protocols target many AP analogues at once. For example, the full LC-MS/MS potential was exploited for the simultaneous determination of six amphetamines and analogues (AP, MA, MDMA, MDA, MDEA and MBDB) in hair, blood and urine [10]. The proposed method used electrospray ionization (ESI) and triple quadrupole (QqQ) instrumentation and proved capable to detect low drug levels in hair, even after single dose intake. More recently, the detection threshold for amphetamines in hair samples has been lowered further. From 20 mg of decontaminated hair, the experimental limits of detection (LODs) were found to range from 0.3 pg/mg for MBDB to 6.3 pg/mg for MDA, while the recommended **cut-off** limit for AP analogues is 200 pg/mg [11]. This method was applied in a real case of MDMA determination, after single ecstasy intake that allegedly occurred before a drug-facilitated crime. Following **segmental analysis** on a victim's strand, proximal and distal hair segments proved negative, while MDMA was detected at 22 pg/mg concentration in the segment corresponding to the offence time period, confirming the occurrence of single exposure to MDMA. Another recent study reported the development of an LC-MS/MS method for the simultaneous screening and determination of a large set (12 substances) of

amphetamine-like anorectics and their metabolites in hair samples [12]. The method used an overnight acidic-methanolic hair extraction, and was applied to real samples from 39 suspected drug abusers, in order to evaluate the diffusion of anorectic and amphetamine (AP, MA, MDMA, and MDA) abuse in Korea. The determination of four amphetamines, together with ketamine and norketamine, in hair was described by Tabernero *et al.* [13]. The high specificity and sensitivity of the LC-MS/MS procedure allowed the simultaneous detection of all target analytes after simple and fast preparation on 20 mg of decontaminated hair, consisting in an ultrasonic extraction in 500 µL of 0.01% formic acid for 4 h followed by direct injection of the extract in the LC-MS/MS system. The method was applied on 25 hair samples taken from subjects known to be ketamine, designer drugs or APs abusers. An even faster extraction of micropulverized hair was recently proposed to detect five amphetamines, plus ketamine, cocaine, and their metabolites, in minute (0.2 mg) hair samples [14]. In this study, high specificity and accurate-mass determination was provided by a linear ion trap-Orbitrap hybrid mass analyzer.

Cocaine and metabolites

Among CNS stimulants, cocaine is certainly the recreational drug most broadly abused. Cocaine, its main metabolite benzoylecgonine (BZE), and other secondary metabolites, such as norcocaine (NC), cocaethylene (CE), and ecgonine methyl ester (EME), are common target analytes considered in hair analysis to monitor habitual cocaine intake [15]. Taking into account the possible sources of external contamination, which are particularly critical for cocaine [16], the introduction of cut-off concentrations for cocaine (500 pg/mg) and its metabolites (50 pg/mg) was suggested [11].

Despite GC-MS methods guarantee acceptable analytical performances for confirmation purposes, improved sensitivity is progressively obtained with LC-MS/MS methods. For example, cocaine and its metabolites BZE, CE and NC were detected in hair at low concentration levels (LODs of 25 pg/mg for all analytes) by means of LC-MS/MS analysis, after solid phase extraction

(SPE) [17]. A highly-selective clean-up process of hair extracts using molecularly imprinted polymers (MIP) was proposed for cocaine and BZE, followed by LC-MS/MS analysis [18]. LODs of 10 pg/mg and 20 pg/mg were estimated for cocaine and BZE respectively, using a QqQ instrument equipped with an ion source operating in atmospheric pressure chemical ionization (APCI) mode. The method was applied to authentic specimens from cocaine users as well as specimens from international proficiency tests [18]. Even better detection and quantitation limits were obtained using a method based on hydrophilic interaction liquid chromatography (HILIC) coupled to MS/MS [19]. The HILIC silica chromatographic column allowed efficient separation of cocaine and all metabolites, including the highly polar EME. By treating 10 mg of hair, experimental LODs of 1 pg/mg or below were found. The detection of ultra-trace amounts of EME in hair provides an additional tool to differentiate passive incorporation from active consumption of cocaine, a crucial issue for this substance [16].

As a matter of fact, the assessment of cocaine abuse by hair analysis is singularly critical, because several factors may induce false positive and false negative results, even if the significance and value of such analytical tool has been strongly underlined [15]. In particular, external contamination [15,20,21], release from cosmetic treatments [21] and interferences from cosmetic and care products [22] has been described. Concentration ratios for cocaine and its main metabolites were determined from hair samples, respectively obtained from habitual cocaine abuses, participants in controlled cocaine administration and externally contaminated blank hair: the results confirmed the common discrimination criteria, based on cut-off and BZE/cocaine concentration ratio [20]. Clear evidence of both cocaine uptake and release, following strong cosmetic treatment is reported in the literature [5,21]. Strong interference in cocaine detection, leading to false negative results, from a common lotion used to prevent hair loss, was demonstrated [22]. However, this interference was observed only when a GC-MS protocol was used, as it originated from trimethylsilyl derivatives of the lotion components, which did not affect LC-MS/MS analysis. In

general, the use of two stages of mass selection considerably reduces the risk of interferences in hair analysis [5].

Opiates

In order to prove heroin consumption through hair analysis, the detection of main metabolites 6-monoacetylmorphine and morphine is generally utilized. As a matter of fact, the simultaneous presence of both metabolites is accepted as evidence to distinguish the active consumption of the drug from possible environmental contamination (or morphine consumption). Since heroin has been for decades a drug of major concern, several effective analytical methods for its detection in hair samples have been described in the past [1-3]. In the last five years, no LC-MS/MS methods were specifically developed for the determination of opiates in hair samples, but these compounds were usually included in multi-target protocols. For this reason, the determination of opiates in hair samples is discussed in the subsequent chapter dedicated to "*Multi-analyte methods for mixed psychoactive substances".*

Cannabinoids

As for cocaine, clear discrimination between passive exposure to Δ^9-tetrahydrocannabinol (THC) and hashish or marijuana abuse is important. Unintentional drug inhalation and adsorption from environmental smoke are frequently claimed to give reason for positive detection of THC, but the detection of its main metabolite 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) in hair usually provides clear evidence of active consumption. However, a cut-off of 50 pg/mg for THC has been proposed for confirmation analyses as a suitable limit to discriminate chronic and occasional use from external contamination [11,23]. Although GC-MS or GC-MS/MS are the techniques of choice for THC and THC-COOH detection in hair, alternative analytical approaches

based on LC-MS/MS instrumentation have been recently proposed [24-26]. Working with 20 mg of decontaminated hair, THC at low concentration levels (LOQ: 10 pg/mg) was determined by a QqQ instrument operating in positive ESI mode after LC separation. The quantitative results were compared with those obtained from immunoassay [24]. In order to monitor chronic cannabis abuse, Mercolini *et al.* developed an LC-MS/MS method for the determination of THC and THC-COOH in hair. The detection of THC-COOH, at concentrations approximately one order-of-magnitude lower than THC, was shown to represent effective evidence for excluding external hair contamination [26]. Remarkably high sensitivity, and low LODs and LOQs (1 pg/mg and 3 pg/mg, respectively, for both analytes) were obtained under selected reaction monitoring (SRM) conditions by operating the ESI source in alternate positive and negative ion modes for the detection of THC and THC-COOH, respectively. Nevertheless, a LOQ of 3 pg/mg for THC-COOH is not yet satisfactory if compared to the cut-off value of 0.2 pg/mg suggested by the SOHT for this metabolite. So far, quantitation limits comparable to this value were obtained only using GC-MS/MS-NCI protocols after sample cleanup by SPE [2]. A radically alternative approach for THC-COOH detection in hair and urine samples was proposed, which combined traditional ESI with surface-activated chemical ionization (SACI) to improve the sensitivity toward the two target analytes [25]. Although different mass analyzers were tested (linear ion trap, triple quadrupole and Orbitrap) with none of them a complete method validation was provided.

Recently, Moosmann *et al.* proposed to detect Δ^9-tetrahydrocannabinolic acid A (THCA-A) as a specific marker for external contamination of hair samples [27]. Previous studies reported that THCA-A detection from hair samples may arise from external contamination brought by sidestream marijuana smoke or contact with cannabis material. An LC-MS/MS method was developed and validated by the authors to detect THCA-A, THC, CBN and CBD and was successfully applied to analyze external contaminated hair samples by marijuana smoke. ESI was operated in the negative ion mode for THCA-A detection and positive ion mode for THC, CBN and CBD [23].

Hallucinogens

In the industrialized countries, the use of hallucinogens for recreational purpose became widespread among drug users and young population in the late sixties. Even now, several natural and synthetic drugs, including phentermine, cathinone, fenfluramine, selegiline, mescaline, 4-bromo-2,5-dimethoxyphenethylamine (2CB), salvinorin A, ibogaine, psilocin, and lysergic acid diethylamide, are quite extensively abused for their hallucinogenic properties. Therefore, the detection of hallucinogens is occasionally requested to toxicology laboratories for forensic investigations [28]. With the exception of few multi-analyte methods [29,30], the common analytical procedures for the detection of hallucinogens in various biological matrices are focused on specific substances, depending on circumstantial evidences [28].

Several procedures using immunoassays, GC-MS, LC-MS or the corresponding tandem mass spectrometric methods (GC-MS/MS or LC-MS/MS) were addressed to the detection of these drugs in the last few years. Most methods limited their investigation to traditional biological fluids (blood and urine) [30-33]. Only few methods were developed to deal with hair samples, so as to provide a reliable investigation tool for long-term abuse or past drug exposure. Some methods adopted GC-MS(/MS) instrumentation to detect hallucinogens in the pg/mg hair concentration range [28]. For example, a GC-MS procedure was described for the simultaneous detection of thirteen psychotropic phenylalkylamine derivatives by Kim et al [29], whereas a GC-MS/MS method using positive chemical ionization was recently adopted for mescaline detection in hair samples [34].

LC-MS/MS was firstly adopted for ibogaine and noribogaine detection in post-mortem hair samples by Cheze *et al* in 2008 [35]. Ibogaine is one of the main indole alkaloid of Tabernanthe Iboga (Apocynaceae) shrub, whose consumption produces deep hallucinations and other psychoactive effects. Its major metabolite, noribogaine, is still active on the CNS. About 50 mg of hair were sonicated in ammonium chloride. After liquid–liquid extraction with a methylene chloride/isopropanol mixture, the organic layer was filtered, evaporated to dryness, and then

reconstituted, before injection. The detection was performed on a QqQ mass spectrometer operating in ESI and SRM data-acquisition modes. The analytical findings from a real post-mortem hair sample usefully supported a drowning death investigation [35].

Lysergic acid diethylamide (LSD) is a powerful hallucinogen derived from lysergic acid, whose detection in biological fluids, and even more in hair samples, is difficult because an extremely low dosage is commonly assumed, resulting in low blood, urine, and hair sample concentrations. Therefore, reliable and highly sensitive LC-MS/MS methods have to be adopted for LSD detection and quantification. Recently, Gaulier *et al.* described the finding of LSD traces in pubic hair of a deceased man who died after a rave-party. About 50 mg of decontaminated hair samples were analyzed with LC-ESI-MS/MS, following a liquid-liquid extraction (LLE) with dichloromethane/ether. An LOD of 0.5 pg/mg was achieved using a QqQ mass spectrometer in SRM acquisition mode [36]. Similarly, Chapuis-Hugon *et al.* detected LSD from hair samples with an LC-ESI-MS/MS procedure. In this case, a selective sample treatment by MIP was adopted, in order to increase method selectivity and sensitivity [37].

Ricard *et al.* presented a procedure addressed to the detection of atropine and scopolamine in hair samples after alleged acute or chronic exposure to *Datura Stramonium*. Illegal use of *Datura* is hardly detected using traditional biological matrices because the half-live of its active principles is short. In contrast, retrospective investigation is allowed by hair analysis, provided that the method is sensitive enough to detect trace amounts of atropine and scopolamine incorporated into the keratin matrix, as in the cited study (LOD = 0.5 pg/mg): the presence of both principles was ascertained all along the analyzed hair strand, after recurring consumption of *Datura Stramonium* [38]. Remarkably, LC-MS/MS methods avoid heat-instability issues occurring for such tropane alkaloids, and therefore provide a better tool for validated quantification in comparison to GC-MS-based methods [28].

Alcohol

Although ethanol is by far the most widely abused psychoactive substance in western Countries, it is generally not considered within the drugs of abuse. Actually, ethanol itself does not represent a target substance in hair analysis, but its metabolites are consistently determined as decisive biomarkers of chronic excessive drinking and abuse [1,2]. As a matter of fact, only one metabolite, ethyl glucuronide, is recurrently but not exclusively determined by LC-MS/MS methods [39-42]. Due to these peculiar features, systematic illustration of the LC-MS/MS procedures developed to determine alcohol biomarkers is out of the scope of the present review, and can be found elsewhere [2,5].

New Psychoactive Substances (NPSs)

In the last years, most forensic laboratories have been challenged worldwide with the need of detecting a variety of new psychoactive substances (NPSs) in different biological specimens, including hair [5]. Since the late 90s, more than 200 NPSs of potential illegal use have been identified, 49 in 2011 alone [43]. These compounds, frequently referred to as either "**designer drugs**" or "legal highs", have little or no previous history of medicinal use. Although the consumption of these drugs has been tolerated for quite a long time, nowadays the majority of them are progressively banned, as long as serious side-effects and acute intoxications (occasionally fatal) are becoming increasingly frequent [44]. However, legal interdiction of specific substances is often frustrated, because their chemical structure can be slightly modified with relative ease to create new and still unbanned drugs with similar properties.

Amphetamine derivatives, with backbone molecular structure of either piperazine or cathinone, represented the initial group of designer drugs. Shortly afterward, another wide group of compounds, known as synthetic cannabinoids, appeared in the black market. Rapid proliferation of new psychoactive substances belonging to these and other classes of drugs followed in the

subsequent years. Although most of the latest drugs act as CNS stimulants, their chemical structures are assorted, ranging from derivatives of pipradrol, ketamine, phencyclidine, arecoline, aminopropylbenzofuran, ring-substituted aminoindans, to thiophenyl bioisosteres of methamphetamine, as well as cocaine-related structures [45]. Over 3000 cannabinoid mimetics were classified into four main classes [46]: (i) analogues of anadamide-related endogenous ligands (N-arachidonylethanolamine); (ii) THC analogues based on dibenzopyran ring, including HU-210; (iii) cyclohexylphenol derivatives, for example CP 47497; and (iv) a miscellaneous group that includes the basic structures of indenes, indoles, and pyrenes, *i.e.*, the JWH series named after the eponymous John W. Huffman.

Although not yet exhaustively studied, it is known that the metabolic transformations of most NPSs, particularly those of the JWH class, is rapid and complex, making the parent drugs absent or hardly detectable in urine samples, *i.e.*, the biological matrix most typically used in forensic toxicology [47]. Even for the few designer-drug metabolites that have been identified, reference standards are often not commercially available. This poses a serious and longstanding problem to toxicological laboratories, which are called to develop analytical procedures to detect their presence in a variety of biological matrices.

Several analytical methods were developed to determine some NPSs and/or their metabolites in either blood or urine [48-57], but no routine urine screening test is generally executed in forensic laboratories to detect them on large population sets. Consequently, almost no prevalence studies are available [58]. For the same reason, the potential association between NPSs use and traffic or occupational accidents is totally unknown so far [47]. A more promising perspective is offered by the determination of NPSs in hair samples, because the parent drugs are mostly present (unlike urine), and long-term retrospective and integrated consumptions can be detected (unlike blood). Nevertheless, only few prevalence studies have been published to date, which describe the protocols for the detection of NPSs in the keratinic matrix and their application to real cohorts of subjects.

In a Letter to the Editor, Torrance and Cooper reported the detection of mephedrone in hair samples at 4.2 and 4.7 ng/mg concentration with an ISO/17025 accredited method, but details on the analytical method used and results interpretation were not included [59]. A specific and accurate method for detection of mephedrone and two metabolites using LC-MS/MS was proposed by Shah *et al.*, which produced an LOQ of 5 pg/mg for mephedrone and 10 pg/mg for its two metabolites. The study reported that among 154 real hair samples analyzed, mephedrone was detected in only 5 samples, while, its metabolites were not detected in any of the analyzed samples [60]. Moreover, mephedrone could be successfully quantified in only one sample, with a concentration of about 21 pg/mg, whereas the other four positive samples had concentrations below the LOQ. These concentrations are so low that they are likely to be due to sporadic drug intake, since in the case of repeated abuse, mephedrone hair concentration is expected to be in the nanogram per milligram range, not pg/mg [5,61].

Gaillard *et al.* developed a specific method to detect meta-chlorophenylpiperazine (mCPP) in hair samples and other autoptic specimens collected from a deceased 20-year-old man. A C18 LC-ESI-MS/MS protocol was adopted to detect mCPP and its isomers (ortho- and para-) with a LOD value of 20 pg/mg. Although the analysis of hair samples provided negative results, conversely to those of urine, bile, liver and humor vitreous specimens, this outcome was useful to confirm other forensic findings, suggesting that mCPP was assumed for the first time by the victim at the time of death [62].

Quantitative analysis of hair samples for 1-benzylpiperazine (BZP) by LC-ESI-MS/MS was described by Bassindale *et al.*, in order to update drug screening methods and verify the consumption and diffusion of such a designer drug among New Zealanders. Remarkably, 14 out of 126 analyzed hair samples were positive to BZP, with concentrations ranging from 0.4 to 33 ng/mg. About 20 mg of decontaminated hair samples were extracted and partially purified by subsequent SPE, prior of instrumental analysis. The mass spectrometric detection was carried out with a QqQ

mass spectrometer operating in positive ion mode, using SRM data acquisition mode for analyte confirmation [63].

A retrospective study on the prevalence of NPSs among hair samples previously tested positive to MDMA or other amphetamines, was conducted by Rust *et al.* [64]. The proposed analytical method screened 325 hair samples for "club drugs", *i.e.*, different groups of designer drugs that are mainly used in clubs or at music events such as raves or festivals, with LODs ranging from 10 pg/mg up to 50 pg/mg. An impressive percentage of positive cases (37%) were ascertained. Even if quantitative data were not reported, the high prevalence found allows one to conclude that, at least the most common NPSs (*e.g.*, meta-chlorophenylpiperazine, mephedrone, and 4-fluoroamphetamine) should be included into the clinical and forensic toxicology screenings within the laboratory routine.

A similar approach of retrospective study on shelved hair samples was carried out for synthetic cannabinoids and recently published [65]. In this study, a UHPLC-MS/MS procedure was developed and validated to determine the possible presence of five synthetic cannabinoids, namely those consistently present in the black market during the period considered (JWH-018, JWH-073, JWH 200, JWH-250 and HU-210), in 179 real hair samples collected from previously proven Cannabis consumers. All tested samples were obtained from subjects undergoing driving re-licensing procedures, supposedly aware of being tested for THC, not for cannabinoid mimetics. Fourteen (7.82%) samples were found positive to at least one synthetic cannabinoid, with concentrations that ranged from 0.50 to 730 pg/mg, again demonstrating significant diffusion among drug abusers [65].

Whilst new illegal substances were progressively introduced into the black market, namely the new generation of synthetic cannabinoids (for example UR-144 and XLR-11), the analytical methods were gradually updated to include the new parent drugs into the screened targets [66]. Figure 1 reports a SRM chromatogram obtained from a real hair sample, which demonstrates multiple drug abuse at relatively high frequency [66]. A wide range of targeted NPSs was

monitored in a recently published procedure for hair analysis [67]. The authors presented a validated LC-MS/MS method for the quantitative determination of 22 synthetic cannabinoids on 50-mg aliquots of human hair. The method was successfully applied to authentic samples obtained from psychiatry patients who had admitted chronic consumption of several herbal mixtures. Eight samples were found positive, with concentrations ranging from the limit of quantification up to 78 pg/mg. Quite curiously, the drug concentrations measured in the hair from these self-declared abusers [67] were significantly lower than those found in two other studies [65,66] conducted on hair of undeclared abusers. The large differences among dose-related pharmacological effects for such a variety of substances open a debate on the possible need of distinct cut-off levels, so as to discriminate chronic consumption from occasional use (or external contamination), going beyond the unique cut-off of 50 pg/mg used for THC in hair [65].

Psychoactive drugs from pharmaceutical products

Hair analysis is not only valuable for the determination of drugs of abuse. In particular, the detection of a variety of **pharmaceutical psychoactive substances** is currently employed in a wide range of clinical and forensic investigations, such as workplace drug testing, driving ability probation, child custody, post-mortem or pre-natal toxicology, therapy compliance control and **drug-facilitated crimes** [68,69]. The present chapter is devoted to the studies in which hair analysis is applied to the detection of the pharmaceutical psychoactive principles that are generally not considered as “drugs of abuse”, even if the borders between the two classes of substances is uncertain. For example, opiates may be legally prescribed as major pain relievers, and, on the other side, commonly prescribed drugs are searched in hair analysis mostly when a suspect of abuse is posed, either in terms of amount or lack of therapeutic purpose.

In some of the cases of toxicological interest, few target compounds have to be detected and accurately quantified in the low pg/mg range, possibly after single dose consumption. More

frequently, the target substances cannot be predicted or the range of compounds potentially consumed is wide, so that multi-target screening methods become necessary to detect as many compounds as possible with a single analysis. In both circumstances, efficient LC-MS/MS analytical protocols have been recently developed, taking advantage of the impressive progresses of modern chromatographic and mass spectrometric techniques. Remarkably, the flexibility of these analytical protocols assures the detection of a wide-range of pharmaceutical products with different chemical structures, or accurate and highly-sensitive determination of specific drugs. In comparison with GC-MS-based methods, simpler and faster preliminary sample treatments can be adopted in the separations based on LC [5].

Several LC-MS/MS methods for analysis of psychoactive pharmaceuticals have been reviewed few years ago [3]. Later and missed LC-MS/MS protocols described in the scientific literature not taken into account by Wada *et al.* are discussed herein [3].

In forensic investigations, determination of one or few psychoactive pharmaceutical substances in hair with LC-MS/MS is usually performed as the second step of an analytical procedure after screening toxicological testing, *e.g.*, with immunochemical assays, GC-MS- or LC-MS-based methods [5]. These LC-MS/MS confirmation protocols are specifically developed and validated to assure precise and accurate quantification of low drug concentrations (low pg/mg range), an essential pre-requisite, for example in segmental analysis, for drug determination possibly after single-dose consumption. Thus, the LC-MS/MS operating conditions, and preliminary hair treatment, are specifically set up to obtain optimal selectivity, detection capability and quantitative precision and accuracy for the analytical method.

Muller *et al.* proposed an LC-MS/MS strategy to detect the tricyclic antidepressant maprotiline, the neuroleptics pipamperone, and citalopram, in hair samples from three cases of alleged suicide. Identification of the three substances was achieved by comparing the experimental MS-data with ESI/CID and MS/MS mass spectral libraries. The authors proposed their flexible LC-MS/MS method as a valuable tool for drug screening [70]. Gaulier *et al.* developed and validated a method

for acepromazine quantification [71]. Johansen *et al.* developed a method to quantify triazolam in hair samples and the analytical protocol was specifically switched from LC-HRMS to LC-MS/MS, in order to increase detection capability and analytical sensitivity [72]. The experimental details for the LC-MS/MS protocols discussed herein are reported in **Table 1**.

Diverse strategies were proposed for the detection of ketamine and norketamine in hair. This toxicological analysis is increasingly requested since ketamine is misused for both recreational purposes and in drug-facilitated sexual assaults, owing to its dissociative and sedative properties. A selective molecularly imprinted solid-phase extraction (MISPE) was proposed for preliminary hair treatment, followed by LC-MS/MS [73]. A hybrid linear ion trap/Orbitrap mass spectrometer was put forward to achieve optimal selectivity in ketamine analysis [14]. An innovative microfluidics HPLC-Chip-MS/MS procedure was developed by Zhu *et al.* to dramatically reduce analysis-time and costs and, consequently, increase the laboratory throughput [74]. It is worth noting that in forensic investigations, all efforts addressed to improve the instrumental performances should always be mediated with the assurance of high quality analytical results, as was stressed in the report of the first international proficiency test for ketamine detection in hair [75].

Unlike most other drugs, γ–hydroxybutyrate (GHB) requires negative ionization mode to obtain optimal sensitivity [76-77]. GHB is a psychoactive depressant drug often misused by young abusers and in drug-facilitated sexual crimes. On the other hand, GHB is a pharmaceutical drug used in the clinical treatment of cataplexy or narcolepsy, alcohol abuse and, recently, in the trial treatment of Alzheimer's cases [76]. Since a limited endogenic production of GHB should always be considered, in forensic casework involving alleged exogenous GHB administration clear discrimination between endogenous and exogenous contributions is crucial, so as the definition of a cut-off value in hair (≤ 2ng/mg) [77].

Hair analysis is becoming an important tool in therapeutic drug monitoring (TDM) for a variety of pharmaceutical substances, when it provides complementary information to blood and urine in compliance investigations. Besides, the reliability of hair segmental analysis is nowadays

recognized, promoting the development and validation of specific and sensitive methods for TDM in hair samples. Doherty *et al.* proposed an LC-MS/MS protocol to detect risperidone, sertraline, paroxetine, trimipramine, mirtazapine, and their metabolites in hair samples [78]. Since excessive consumption of these commonly prescribed antidepressants may lead to suicide and other potential side-effects, the accurate determination of parent compounds and major active metabolites in hair samples is occasionally necessary for long-term retrospective investigations. In the cited study, extensive use of low- and high-resolution MS/MS provided definite fragmentation pathways and optimal SRM transitions for the final arrangement of the confirmation test [78]. A similar strategy was adopted also for venlafaxine, fluoxetine and citalopram detection [79].

Shortly after the introduction of rimonabant as the first cannabinoid antagonist licensed in Europe for the treatment of obesity (now withdrawn from the market), an LC-MS/MS protocol for its detection in human plasma and hair was published, allowing to ascertain daily and long-term compliance/intake [80]. Similar strategies were followed for (i) clozapine, to elucidate a unauthorized long-term administration on psychiatric patients [81]; (ii) risperidone an anti-psychotic drug of second generation [82]; and (iii) atomoxetine, a non-stimulant drug approved for the treatment of attention-deficit hyperactivity disorder (ADHD) in pediatric and non-pediatric patients as an alternative to methylphenidate (MPH) [83].

Whenever a drug is administered in maintenance programs, under controlled conditions, hair analysis represents an opportunity to verify compliance to the prescribed dosage, provided that a direct relationship between daily dosage and hair concentration has been verified, which is not always the case. For example, Papaseit *et al.* proved that no linear correlation exists between the administered atomoxetine daily dosage and hair concentrations [83]. Goodwin *et al.* investigated the relation between dose of buprenorphine assumed by pregnant women and maternal and infant hair concentrations, which showed significant relationship between buprenorphine dose and maternal hair concentrations, but no correlation with the infant hair, and resulted in the chance of qualitative, not quantitative, verification of drug-exposure during gestation [84]. Skopp *et al.* reported

significant correlation between oral buprenorphine dosage and the detected hair concentration of buprenorphine and norbuprenorphine, even if high inter-individual variability was observed [85]. In general, the occasional lack of correlation between dosage and hair concentration, observed for some drugs, is possibly due to the heterogeneous nature of keratin, the potential impact of external contaminations or passive exposure, the occurrence of cosmetic treatments, and individual differences in drug metabolism or incorporation rate. Therefore, while it is generally agreed that qualitative results from hair analysis are always valid, the interpretation of quantitative results is still under debate for many drugs [86]. Strategies, such as the detection of drug metabolites, are applied to reduce the inter-individual variability in parent drug incorporation, to support positive findings and minimize the chance of external contaminations and passive exposure, although they are rarely utilized as absolute discrimination tools [85-87].

Another relevant field of toxicological investigation is represented by the specific identification and quantification of chiral pharmaceutical compounds in diverse biological matrices, including hair. The application of various analytical protocols for enantiomer detection and discrimination has been recently reviewed [88]. It was assessed that the hyphenation of LC and MS/MS frequently represents an ideal tool to identify enantiomers, characterized by nearly identical chemical properties, but only one of the cited papers refers to hair analysis [88]. This concerns the identification of methadone enantiomers and their main chiral metabolites EDDP and EMDP [89]. Racemic methadone was administered to former heroin abusers undergoing maintenance therapy, but the (R)-enantiomer is almost exclusively responsible for the analgesic and abstinence relieving effects. A multivariate design of experiments was used to optimize the chromatographic separation that proved essential for enantiomer distinction, since the same SRM transitions were followed for methadone and metabolite enantiomer detection [89]. Another successful study of chiral separation in hair analysis refers to the identification of dextromethorphan and levomethorphan [90]. Dextromethorphan is widely used as an antitussive medicine, showing dissociative and hallucinogenic effects in large dosage misuse. Its enantiomer levomethorphan is a potent narcotic

analgesic that has never been used for therapeutic purposes. To verify the possible adulteration and substitution of dextromethorphan with levomethorphan for illegal purposes, it is necessary to establish their distinction in biological samples by means of enantiomeric separation. In the cited study, rat hair samples were analyzed, in order to prove drug incorporation into the keratin matrix, and highlight the differences in the metabolic pathways for the two enantiomers, from the concentration ratio between parent drugs and their main metabolites. The analytical protocol was prospectively proposed for human hair sample analysis [90].

Benzodiazepines and "z-drugs"

Benzodiazepines and few other psychoactive drugs not belonging to the class of benzodiazepines, namely "z-drugs" (zolpidem, zopiclone, zaleplon), are the most frequently and widely prescribed sedative, tranquilizer and hypnotic drugs worldwide. Dozens of benzodiazepines with unquestionable effectiveness have been introduced in the clinical area for the treatment of various sleep and psychiatric disorders. Despite their beneficial use as therapeutic drugs, they are subjected to possible abuse and controlled in many countries, due to their inherent ability to cause tolerance and addiction - especially in combination with alcohol [91] - or severe intoxication at high dose [92].

Misuse of benzodiazepines is occasionally exploited to perpetrate drug-facilitated crimes, including robberies and sexual assaults, on victims made compliant by the effects of the drug. Due to their similar effects, it is unlikely that the specific drug used in drug-facilitated crimes could be determined only by symptoms [93]. Therefore, the availability of effective LC-MS/MS methods addressed to the simultaneous detection and quantification of several benzodiazepines and "z-drugs" is mandatory for conducting such investigations. Remarkably, some benzodiazepines interconvert into one another during the metabolic processes (*e.g.*, from diazepam, nordiazepam and

temazepam are produced, the latter evolving further to noxazepam [92]), a feature that encourages the adoption of multi-target methods.

An LC-MS/MS method was developed and validated by Xiang *et al.* for the determination of 18 benzodiazepines in hair [94]. A 30-min chromatographic separation was obtained with pentafluorophenyl propyl column, followed by positive ion ESI and SRM detection on a QqQ MS. From 20-mg hair aliquots, detection limits ranging between 0.2 pg/mg (estazolam) and 5.0 pg/mg were observed, which proved adequate to detect the target benzodiazepines after single dose intake. Hair samples were analyzed from 14 volunteers who assumed single 1-4 mg dose of estazolam and from two victims of sexual crimes. All 0-2 cm proximal segments were found positive for estazolam, and the relationship between hair concentration and dosage proved significant, with narrow inter-individual variations at the same dosage (less 40%). Clonazepam and 7-aminoclonazepam were detected in the 0-2 cm proximal hair segment from the two victims of sexual assault, at concentrations ranging from units to tens of pg/mg [94].

A multi-target LC-MS/MS method for the detection of 21 benzodiazepines and 3 “z-drugs” in hair samples was recently proposed [91], which involved a 14-min chromatographic separation, positive ESI, and combined SRM (three transitions) and enhanced product ion scan (EPI) mass spectrometric detection, using a hybrid triple quadrupole/linear ion trap instrument. Highly reliable compound identification was allowed by this new generation of mass spectrometers operating in the dependent acquisition (IDA) mode. Low quantification limits were achieved from 30-mg hair aliquots, ranging from 0.6 pg/mg to 10 pg/mg. The new method was tested on 175 real hair samples, 54% of which proved positive for at least one target analytes, with highest frequency for zolpidem, diazepam, nordazepam and oxazepam [91].

Another method aimed to the detection of 27 benzodiazepines and metabolites, together with zolpidem, was developed and validated for forensic applications [92], again using a hybrid triple quadrupole/linear ion trap mass spectrometer. The proposed analytical protocol proved to gain extremely low detection limits, which ranged from 0.005 ng (zolpidem) to 0.5 ng (bromazepam) in

10-mg hair samples. The method was applied to the detection of diazepam (and metabolites) and lormetazepam in hair samples from rats after single- and multiple-dose administration [92]. Subsequently, 18 criminal cases involving the illegal use of benzodiazepines and zolpidem were substantiated by hair analysis using the same analytical protocol [95].

Similar procedure and instrumental setup was used in a recent study that compared two extraction procedures to detect 35 benzodiazepines, metabolites and "z-drugs" from hair samples [69]. In the first procedure, about 20 mg of decontaminated hair were sonicated and then extracted with dichloromethane/diethyl ester. The organic solvent was dried under nitrogen and the residue reconstituted in methanol before LC-MS/MS analysis. In the second procedure, the hair sample was extracted with methanol and the organic phase was directly analyzed. Both methods were fully validated. As expected, the first procedure proved more sensitive (LOD values ranging from 0.1 to 1.0 pg/mg) than the second one (LOD values ranging from 0.1 to 5.0 pg/mg), but detection and quantification limits were low enough to detect therapeutic concentrations with both procedures. The two procedures were applied to analyze 35 real hair samples, including post-mortem cases and healthy volunteers who declared therapeutic use of lorazepam: 15 out of 35 cases tested positive for one or more substances [69].

Salomone *et al.* introduced a method devoted to the detection of the most frequently prescribed benzodiazepines and "z-drugs" in Italy plus ketamine and scopolamine, which was specifically designed to analyze hair samples arising from cases of suspected drug-facilitated sexual crimes. 14 substances were chromatographically separated and detected in 22 min with an LC-ESI-MS/MS instrument carrying out three SRM transitions for each analyte [93]. LOD values between 0.2 and 4.0 pg/mg were obtained for the target analytes. The method was applied to investigate the case of two women sexually abused by the same man, under the incapacitating effect of zolpidem. Accurate segmental analysis was adopted to correlate analytical results, chronological reenactment and other forensic evidences, so that the assumption of single dose administration was substantiated with the detection of the drug at trace level in the time-matching segment, with no presence in the preceding

and subsequent segments. Scanning electron microscopy (SEM) was used to investigate the state and conservation of the hair cuticle several months after the reported episodes [93]. Indeed, it is known that external contamination or partial loss of the drug and/or displacement by radial migration after incorporation into the keratin matrix can occur when the hair structure is deteriorated by thermal and/or cosmetic treatments. On the other hand, broadening of xenobiotics distribution along the hair length may be produced during the resting period of hair growth [1].

Multi-analyte methods for mixed psychoactive substances

The analytical methods reviewed in the previous sections were designed to be specific for a particular class of drugs. Consequently, optimization of sample treatment, clean-up and chromatographic conditions represented the primary objective to meet accuracy, sensitivity and specificity acceptance criteria, leading to reliable confirmation results. This strategy is no more effective when a wide screening of abused drug has to be done, because in such a case the use of several class-specific procedures would be needed, with direct impact on efficiency and costs. Conversely, screening on large cohorts of subjects, such as for example in workplace drug testing, requires rapid, cheap, and widescreen procedures, even to the detriment of optimal sensitivity and accuracy. For this reasons, the extensive multi-target capability of LC-MS/MS instrumentation is increasingly employed to develop protocols for general drug screening, after non-selective extraction procedures, which are adequate for analytes with widely different physico-chemical properties.

For example, a fast and sensitive LC-ESI-MS/MS method was developed by Huang *et al.* for the simultaneous determination of opiates, cocaine and their metabolites, but not THC (due to its peculiar chemical properties) nor amphetamines [96]. The extraction was done from 20 mg of pulverized hair by ultrasonication in methanol. This validated method was applied on 79 authentic hair samples from drug abusers, from which a correlation between cocaine and BZE content in hair

segments was established, allowing to clearly discriminate active consumption from external contamination. Similarly, an ion trap MS was used in combination with LC to screen 16 drugs of abuse, including cocaine, opiates, amphetamines, and other stimulants in human hair [97]. The analysis was carried out with two separate injections, in which the mode of data acquisition was switched between MS/MS screening and MS^3 confirmation. A further method devoted to the detection of opiates, cocaine, amphetamines and metabolites (11 substances) made use of APCI source, instead of ESI [98]. After a simple methanolic extraction of hair samples, this LC-MS/MS method exhibited sufficient sensitivity to be applied for segmental analysis (LOQs values: 200 pg/mg for opiates and amphetamines, 50 pg/mg for cocainics). The validated procedure was applied for the retrospective multi-parameter evaluation and distribution of opiates in hair samples originating from heroin abusers [98].

Another group of multi-target LC-MS/MS methods was developed with the aim of including some common benzodiazepines among the target drugs of abuse. For example, a procedure for screening 22 drugs of abuse in hair samples, excluding THC but comprising six benzodiazepines and two “z-drugs” (zolpidem and zopiclone), was developed by Hegstad *et al.* [99]. Only one transition was monitored for each compound on the QqQ mass spectrometer, so as to improve scan speed and sensitivity, resulting in LOQs within the interval 5-50 pg/mg for the most common drugs of abuse. More recently, a wider LC-MS/MS screening of 35 substances in hair, that included THC, the most common drugs of abuse (opiates, amphetamines, cocaine, LSD, ketamine and scopolamine) and several pharmaceutical drugs (benzodiazepines, antidepressants and hypnotics), was proposed by Lendoiro *et al*. [68]. An extraction procedure involving two steps (LLE followed by SPE) was performed on 50-mg decontaminated hair aliquots. As for the previous study, only one transition per compound was monitored in SRM mode during the screening phase, whereas the non-negative results were subjected to confirmation by a second LC-MS/MS run, in which the acquisition protocol was integrated with an additional transition for each compound to be

confirmed. For the entire range of investigated molecules, the experimental LODs were in the range 0.2-50 pg/mg, largely meeting the Society of Hair Testing (SOHT) cut-offs requirements [68].

The use of tridimensional ion-traps in broad toxicological screenings offers the advantage of easy full scan MS/MS (product ion) spectra acquisition. On the other hand, restrictions arise from the limited number of scan events that can be monitored simultaneously. Miller *et al.* programmed three separate LC-MS/MS injections to determine cocaine, opiates, amphetamines, diazepam and their metabolites in hair samples using an ion trap MS operating in ESI [100]. After single incubation of hair samples in phosphate buffer (pH 5.0) and subsequent SPE extraction, 17 substances could be progressively screened, although the sensitivity was lower (LOQs: 50-310 pg/mg) than that achievable with QqQ instruments [100]. Using an ion trap as mass spectrometer with ESI source operating in the positive ion mode, a general screening of unknown drugs in biological specimens, including hair, was developed by Liu *et al.* [101]. An in-house spectral library of approximately 800 substances was created by the authors and used only for analytes' identification.

Finally, the recent introduction of UHPLC allows the separation and detection of a wide variety of substances of toxicological interest in a single run of only few minutes. The combination of UHPLC with triple quadrupole MS/MS was recently adopted for the simultaneous screening of thirteen analytes in hair samples, including opiates, cocaine, amphetamines, THC, buprenorphine, methadone and a few of their metabolites [102]. Using a simple sample preparation based on methanolic extraction, excellent sensitivity and selectivity were achieved with LOD values in the interval 6-27 pg/mg [102].

Systematic toxicological analysis

In several clinical and forensic investigations, including suicides, chronic criminal administration of drugs or poisons (*e.g.*, for sedation of children and non-self sufficient elderly persons),

recreational drug abuse, death cases after chronic medical treatment and clinical cases with unknown history, a broad spectrum general search for toxic substances that cannot be foreseen, called systematic toxicological analysis (STA), is requested. Specific features of STA conducted on hair samples comprise (i) a simple and non-selective extraction procedure, which recovers as many toxicologically relevant substances as possible and excludes most matrix constituents; (ii) chromatographic separation of the extract components under non-specific conditions and (iii) identification and quantification of relevant components by means of MS [5,103]. Frequently, full-scan MS experiments with high-resolution mass analyzers are adopted for STA screening methods, so that the identification of unknown substances can be guided by exact-mass data [104-106]. Time-of-flight (TOF) mass spectrometers with progressively increased mass resolution and accuracy offer the advantage of obtaining molecular formula, isotopic distribution, and comprehensive mass pattern from all components of the injected hair extract without any pre-determination, and with the chance of delayed re-evaluation. Nevertheless, MS/MS experiments can still increase the substance identification ability [103]; in particular, hybrid quadrupole-TOF-MS provide the collection of accurate mass fragment ion spectra induced by collisional activation [104], so that unambiguous identification of unknown substances is conducted through the comparison of potential candidates against theoretical and experimental libraries of toxic compounds containing their retention times and product-ion tandem mass spectra (protocols similar to those used in metabolomics) [105,107].

In order to acquire exhaustive data collection for later examination, Broecker *et al.* operated the instrument in steady alternation between MS and MS/MS modes, the latter collecting product-ion spectra from two precursor ions at variable and mass dependent collision energy. The method was adopted for the analysis of real hair samples from 90 death cases with known exposure to illegal or therapeutic drugs during life time [103]. Overall, 154 parent drugs and 28 metabolites were identified. The psychoactive molecules most frequently detected included 17 antidepressants, 15 neuroleptics, 11 drugs for the treatment of cardiovascular diseases, 10 opioid analgesics, 8 non-opioid analgesics, 7 benzodiazepines, 6 anti-epileptics, 6 antihistaminics, 5 hypnotics, 13 antibiotics

and 7 fungicides. LOD values ranged from 1 pg/mg to 10 pg/mg. It is worth noting that the results were compared with case histories from police reports and the results of toxicological analysis from blood, urine and gastric content; it was found that only 40% of the taken drugs were identified also in hair. The possible reasons for this deceptive negative result in hair analysis were extensively appraised by the authors, and further methodological improvements were suggested [103].

Conclusions

The primary adoption of LC-MS/MS procedures in the clinical and toxicological investigations involving hair samples is nowadays universally recognized, since this instrumentation guarantees minimal sample treatment, low detection capability, precise and accurate quantification, and high laboratory throughput, in both class-specific and multi-class inquiries. The ever-increasing performances of LC-MS/MS methods already provide minimal LOD and LOQ values, that largely beat the concentrations expected in real hair samples, even after single drug administration.

LC-MS/MS guarantees extreme flexibility in the detection of psychoactive substances, which range from non-polar to extremely polar, from acidic to basic, and from low to high molecular weight. This analytical feature also allows one to separate chiral substances, to accomplish ultimate specificity or, conversely, to determine hundreds of compounds at once. This impressive flexibility is the result of variable combinations of chromatographic conditions, ionization techniques and data-acquisition modes, together with fast electronic control of the mass analyzers.

The cited features of LC-MS/MS explain why the existing GC-MS methods are progressively substituted in toxicological and clinical hair analysis. GC-MS is still extensively used when single substances, or a restricted panel of substances, have to be accurately determined. The GC-MS approach is no more advisable whenever (1) wide-range or general toxicological screening have to be executed (for example, in acute intoxications and post-mortem investigations), (2) a large number of samples has to be processed in a short time (for example, in workplace drug testing), (3)

ultimate sensitivity has to be reached (*i.e.*, after single dose administration; in segmental hair analysis; in pre-natal exposure testing), (4) parent drugs and their polar metabolites have to be simultaneously determined. In all these situations, UHPLC-MS/MS already represents the gold standard for hair analysis.

Future Perspectives

As long as hair analysis with LC-MS/MS is increasingly requested for a variety of clinical and forensic investigations to be ultimately included within the routine workflow, it can be envisioned that further technology improvements in sample handling automation, robotics and SPE-LC-MS/MS coupling will dramatically reduce the time requested for hair sample processing. In parallel with the introduction of further generations of UHPLC instruments, able to reduce the chromatographic run time, new "multiplex" LC-MS/MS strategies have already been proposed for routine toxicological analysis on urine or blood samples [108,109]. In multiplexing setup, multiple LC columns are connected in parallel with one mass analyzer, each loaded with a different sample, and operating so as the eluates reach the MS system in sequence, without overlapping. This configuration is particularly useful for confirmation methods, when target compounds are generally eluted within short time intervals during which the mass spectrometer is requested to acquire data; then, the eluate is diverted and the next column is interfaced to the MS [109].

On the other hand, multi-class and multi-target LC-MS/MS methods, performed with QqQ instruments, keep enlarging the range of substances that can be simultaneously determined, with increasing sensitivity. This expanded capability, already effective in blood analysis will become common also in hair analysis, as long as effective extraction condition will be established for compounds with extremely variable chemical properties. Altogether, these technological improvements will substantially contribute to the economical sustainability of complex and widely applied toxicological analyses on hair samples.

TOF-MS and hybrid Q-TOF instruments will be increasingly utilized for general and systematic toxicological analysis, especially in post-mortem investigations, when the causes of death are often unpredictable. High-resolution mass determination and full-scan acquisition, typical of TOF/MS, represent essential features for such inquiries.

Although direct MS analyses by means of DESI, MALDI-TOF and MS-imaging techniques, combined with simplified sample handling, have been recently proposed as fast and cheap alternative procedures to chromatographic-mass spectrometric methods for hair analysis [5], these techniques are still applicable for rough qualitative screening only, and far from replacing LC-MS/MS for accurate quantification and trustworthy validation.

Lastly, but most importantly, future research will progressively focus on the reliable and conclusive interpretation of analytical results [21]. Indeed, hair is still a matrix under comprehensive examination, because several features of both drug incorporation and release have not completely clarified yet. Individual metabolic variability, keratin permeability, hair pigmentation and thickness, environmental and auto-contamination, longitudinal diffusion, effects of cosmetic treatments and repeated washing are altogether crucial aspects to be considered in systematic studies, before hair analysis could be considered as reliable and predictable as any other toxicological and bioanalytical investigation.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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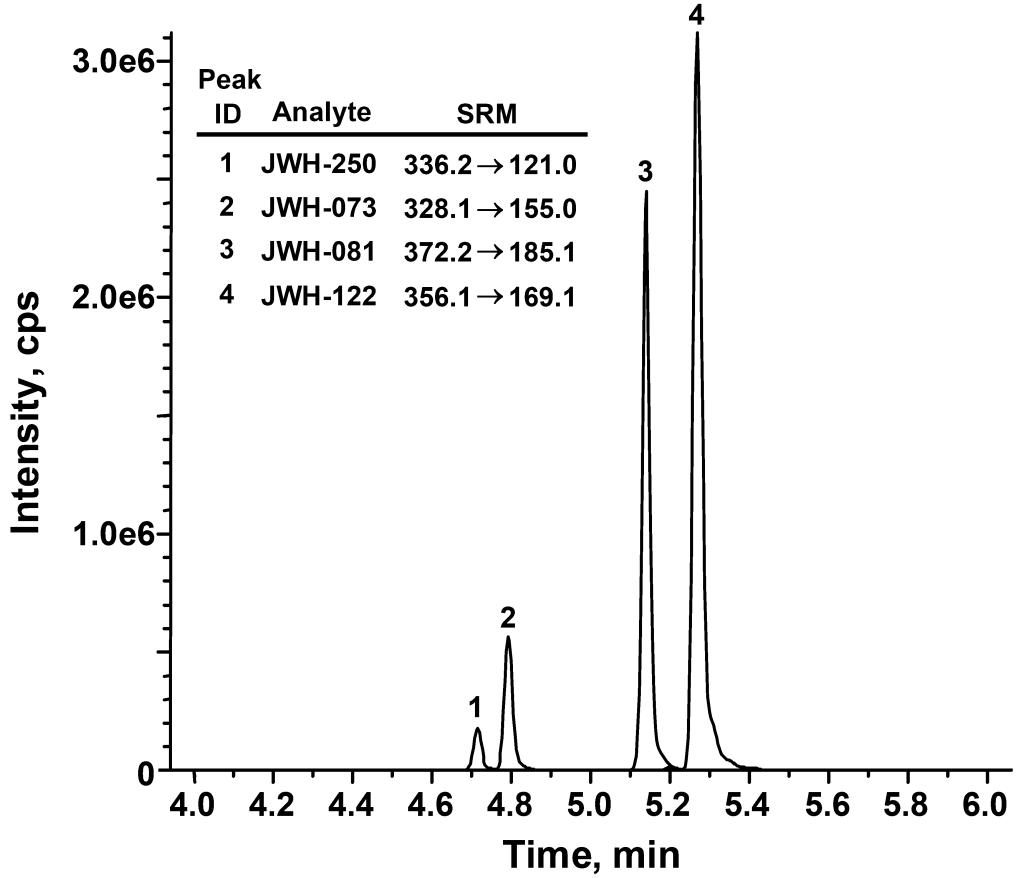

Figure 1. ESI+ SRM chromatogram obtained from a real hair sample, which demonstrates multiple synthetic cannabinoid abuse. Each analyte is labeled by the progressive number assigned as in the inset. Only the target ion is shown for each analyte.

Table 1 Analytical procedure for psychoactive pharmaceutical drug detection in hair samples

Target	Instrument	Pretreatment	Hair amount used (mg)	LOD (pg/mg)	Reference
Acepromazine	X-Terra MS C18 (2.1×50 mm, particle size 5 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Extraction with NaOH followed by SPE pre-separation step	50	5	[71]
Atomoxetine and metabolites	YMC-Pack TMS (4.6×40 mm; particle size 5-3 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Overnight incubation with NaOH. LLE with tert-butyl methyl ether. Evaporation to dryness and re-dissolvation in LC mobile phase	20	50; 60	[83]
Buprenorphine; norbuprenorphine	YMC ODS-AQ (2.0×150 mm, S-3 120 Å). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Overnight digestion with NaOH. LLE with ethylacetate/acetonitrile/n-butyl chloride	1.6-10.1	3.0 (LOQ)	[84]
	Zorbax Ecliple XDB 8 (2.1×150 mm, particle size 5 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Extraction with methanol and incubation overnight at 40°C. Evaporation to dryness, re-dissolution in NaOH and chlorobutane/acetonitrile	40	3; 4	[85]
Clozapine; norclozapine	Synergy Polar-RP (2.0×75 mm, particle size 4 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Ultrasonication with methanol for 3 h. Evaporation to dryness and re-dissolvation in LC mobile phase	50	n.a.*	[81]
Dextromethorphan; levomethorphan and metabolites	Chiral CD-Ph (2.1×150 mm, particle size 5 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Liquid extraction with methanol and HCl. Filtration and evaporation to dryness. Re-dissolvation in distilled water	10	25	[90]

*data not available

Table 1 (continued)

Target	Instrument	Pretreatment	Hair amount used (mg)	LOD (pg/mg)	Reference
GHB	Zorbax SB-C18 (2.1×50 mm, particle size 1.8 µm). Triple quadrupole MS (ESI source in negative ion mode). SRM data-acquisition mode	NaOH digestion at 75° for 40 min. Acidification and addiction of ethyl acetate. Separation of supernatant layer, evaporation to dryness and reconstitution with LC mobile phase	25	500	[76]
	Phenomenex Luna C18 (2.0×100 mm, particle size 5 µm). Triple quadrupole MS (APCI source in negative ion mode). SRM data-acquisition mode	Hair digestion with NaOH. Addition of sulfuric acid and ethyl acetate. Evaporation of supernatant phase to dryness and re-dissolvation with LC mobile phase	25	200	[77]
Ketamine; norketamine	Unison UK-C18 (3.0×150 mm). Hybrid linear ion trap-Orbitrap MS (ESI source in positive ion mode). SRM data-acquisition mode)	Micropulverized extraction with ammonium formate and formic acid. Filtration of the suspension with a 0.45 µm PDVF microporous membrane	0.2	50; 100	[14]
	Synergi Hydro RP (2.0×150 mm, particle size 4 µm). Ion trap MS (ESI source in positive ion mode). SRM data-acquisition mode	Overnight incubation with phosphate buffer. MISPE extraction procedure with acetonitrile and acetic acid. Evaporation to dryness and re-dissolvation in LC mobile phase	10	100; 140	[73]
	HPLC polymeric Chip inserted into the HPLC-Chip–MS/MS interface: a 25 mm, 500 nL enrichment column packed with ZORBAX 80 SB-C18 (0.75×150 mm, particle size 5 µm) and a separation column packed with ZORBAX 80 SB-C18 (0.75×150 mm, particle size 5 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Incubation with HCl for 4 h, followed by neutralization with NaOH and phosphate buffer. Liqui liquid extraction with dichloromethane/hexane. Evaporation to dryness and re-dissolvation in water/acetonitrile. The sample was diluted 100-fold with the same solvent before the analysis. 2-µL aliquot of each solution was injected into the HPLC-Chip–MS/MS system for analysis transferred to a conical insert with polymer feet in a glass vial.	10	0.5; 0.5	[74]

Table 1 (continued)

Target	Instrument	Pretreatment	Hair amount used (mg)	LOD (pg/mg)	Reference
Methadone; EDDP	X-Terra MS C18 (2.1×100 mm, particle size 3.5 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Overnight incubation with ammonium buffer. Liquid liquid extraction with dichloromethane/isopropanol/ heptane. Evaporation to dryness and re-dissolution in acetonitrile/formate buffer	20	2; 2	[86]
Methadone; EDDP; EMDP	Chiral HPLC column using an α-glycoprotein (AGP) stationary phase (4.0×150 mm, particle size 5 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Overnight incubation with methanol and TFA. Evaporation to dryness and re-dissolvation in MilliQ water	75	1.5; 8; 90	[89]
Pipamperone; maprotiline; citalopram	RP-C8 (4.0×125 mm, particle size 5 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Ultrasonication with methanol for 2 h. Evaporation to dryness of the organic phase and re-dissolvation in phosphate buffer. SPE purification. Re-dissolvation of the evaporated eluate in LC mobile phase	50	100 (LOQ)	[70]
Rimonabant	Hypurity C18 ThermoHypersil (2.1×150 mm, particle size 5 µm). Ion trap MS (ESI source in positive ion mode). MRM data-acquisition mode	Overnight incubation with carbonate buffer. LLE with diethylether. Evaporation to dryness and re-dissolvation in LC mobile phase	10	2.5 (LOQ)	[80]
Risperidone; sertraline; paroxetine trimipramine; mirtazapine and metabolites	Phenomenex C18 (4.6×150 mm, particle size 5 µm). Hybrid QTrap MS (ESI source in positive ion mode). MRM data-acquisition mode	Soxhlet extraction with methanol at 160°C. Evaporation to dryness and re-dissolvation with LC mobile phase	105-200	n.a.*	[78]

*data not available

Table 1 (continued)

Target	Instrument	Pretreatment	Hair amount used (mg)	LOD (pg/mg)	Reference
Risperidone; 9-hydroxyrisperidone	X-Terra MS C18 (3.9×150 mm, particle size 5 µm). Ion trap MS (ESI source in positive ion mode). SRM data-acquisition mode	Phosphate incubation pH 9.5. LLE with hexane/ dichloromethane/ isopropanol. Separation and evaporation to dryness of the organic layer. Reconstitution with LC mobile phase	n.a.*	0.9; 1.5	[82]
Tramadol; N-desmethyltramadol; O-desmethyltramadol;	Kinetex C18 (2.1×50 mm, particle size 2.6 µm). Hybrid QTrap MS (ESI source in positive ion mode). MRM data-acquisition mode	Two step extraction with methanol and HCl/methanolic solution. Evaporation to dryness, re-dissolution in methanol and ammonium formate	20	5; 10; 5 (LOQ)	[87]
Triazolam; α-hydroxytriazolam	BEH C18 UPLC column (2.1×100 mm, 1.7 µm). Triple quadrupole MS (ESI source in positive ion mode). SRM data-acquisition mode	Liquid extraction with methanol/acetonitrile/ammonium formate, followed by centrifugation. The solution was transferred to mini-UniPrep vials containing PTFE filter. Filtered extract was injected onto the UPLCMS/MS	20	0.1; 0.5	[72]