Received: 27 September 2019 | Revised: 31 January 2020 | Accepted: 8 February 2020

DOI: 10.1002/jat.3959

HYPOTHESIS REVIEW/THEORETICAL ARTICLE

Journal of Applied Toxicology WILEY

Variability associated with interpreting drugs within forensic hair analysis: A three-stage interpretation

Christopher Davies[1] | Lata Gautam[1] | Agatha Grela[2] | Joanne Morrissey[1]

[1]Forensic and Investigative Studies, School of Life Science, Faculty of Science and Engineering, Anglia Ruskin University, Cambridge, UK

[2]School of Human and Social Sciences, University of West London, Brentford, London, UK

Correspondence
Christopher Davies, Forensic and Investigative Studies, School of Life Science, Faculty of Science and Engineering, Anglia Ruskin University, Cambridge CB1 1PT, UK.
Email: christopher.davies3@pgr.anglia.ac.uk

Abstract

Hair analysis is capable of determining both an individual's long-term drug history and a single exposure to a drug, which can be particularly important for corroborating incidents of drug-facilitated crimes. As a source of forensic evidence that may be used in a court of law, it must be credible, impartial and reliable, yet the pathways of drug and metabolite entry into hair are still uncertain. Many variables may influence drug analysis results, most of which are outside of the control of an analyst. An individual's pharmacokinetic and metabolic responses, hair growth rates, drug incorporation routes, axial migration, ethnicity, age and gender, for example, all display interpersonal variability. At present there is little standardization of the analytical processes involved with hair analysis. Both false positives and negative results for drugs are frequently encountered, regardless of whether a person has consumed a drug or not. In this regard, we have categorized these variables and proposed a three-stage analytical approach to facilitate forensic toxicologists, hair analysis experts, judiciaries and service users in the analytical and interpretation process.

KEYWORDS

axial migration, data interpretation, drug-facilitated sexual assault, drug hair analysis, hair growth rate, segmental analysis

1 | INTRODUCTION

Hair analysis has become a powerful biological sample within forensic toxicology for determining both single and long-term exposure to drugs. It has particularly revealed its usefulness for the detection of drugs and metabolites when corroborating incidents of drug-facilitated crimes (DFCs) and drug-facilitated sexual assaults (DFSA) (Chatterton & Kintz, 2014; Kim et al., 2015; Kim, In, Choi, & Lee, 2013; Rossi, Lancia, Gambelunghe, Oliva, & Fucci, 2009).

Many drugs associated with DFC and DFSA exhibit short metabolic half-lives, being rapidly removed from the body, and becoming quickly undetectable (Carter, 2010; Gautam, Sharratt, & Cole, 2014; Kintz, Villain, Dumestre-Toulet, & Ludes, 2005). Therefore, blood and urine sampling only offers a short-term window for their detection, which may lead to DFC claims being inconclusive, most notably as many alleged DFSA victims are unaware of, or reluctant to come forward, and may delay the reporting of a possible crime (Concheiro et al., 2005; Hurley, Parker, & Wells, 2006; Madea & Mußhoff, 2009). Hair sampling is less invasive than blood and urine sampling and can add significant evidential weight to investigations by widening the detection window from a few hours to many months. Nevertheless, there are gaps in scientific knowledge that can hinder the reliability of this potentially powerful source of evidence.

As a forensic tool that may be used in a court of law, hair analysis must be just as accurate and as reliable as any other type of evidence, yet there is little consensus, standardization or agreement on the analytical processes, making the comparison of data between laboratories challenging. Many variables may interfere with the outcome of results that can be easily overlooked. Data analysis should recognize variables beyond the control of the analytical process, which may include the individual's pharmacokinetic and metabolic responses to a drug (Carter, 2010), hair growth rates (LeBeau, Montgomery, & Brewer,

2011), drug incorporation routes (Harkey, 1993; Polettini, Cone, Gorelick, & Huestis, 2012), axial migration (Kintz, 2013) and hair anatomy differences between ethnicity, age and gender (Gibson & Skett, 2001). Many of these variables are outside of the control of an analyst, leading to the introduction of unavoidable mediator variables. Therefore, it is important to be cautious when interpreting any hair analysis data, and to apply knowledge of whether these variables may have affected the data obtained.

In this paper, we discuss the current level of understanding of these variables, raising awareness of important considerations for data interpretation. We have categorized these variables into three stages that may influence the detection of drugs within hair analysis (Table 1). The variables in each stage have been grouped in a way that their properties will influence the subsequent stages. While stage 1 influences stage 2, and stage 2 influences stage 3, the only variables that are within the control of the analyst are within the third, i.e., final stage. Therefore, these stages should be considered when interpreting the results of the drug hair analysis, and the significance of variables expressed within the findings.

2 | STAGE 1 VARIABLES: DRUG PHARMACOKINETIC INTERACTION

2.1 | Dose and drug metabolism

The pharmacokinetic parameters of drugs and how they interact with the body has been widely investigated, and has been shown to be interperson specific, with varying responses, rates of metabolism and quantities of metabolites produced (Taxak & Bharatam, 2014). The pharmacology of these is far beyond the scope of this paper. Nevertheless, it is important to consider these uncontrollable variables when interpreting the results of any hair analysis.

A correlation between the administered dosage of a drug and the detection of its corresponding metabolites within hair has yet to be proven for an entire sample population.

A male and female volunteer were each administered single 6 mg doses of bromazepam (Villain, Chèze, Dumestre, Ludes, & Kintz, 2004). Bromazepam was detected at 0.8 and 4.7 pg/mg respectively in the proximal 0-2 cm segments. In a similar study, Chèze, Villain, and Pépin (2004) detected 3.5 pg/mg bromazepam (6 mg single dose) in the first 1 cm proximal segment. The study was repeated by powdering hair samples, which increased the detection of bromazepam to 28 pg/mg. The difference is most likely attributed to greater extraction due to leaching of the drug from the increased hair surface area. The dose consumed, hair washing and analytical technique used were the same in these studies.

In two other notable studies, volunteers were administered 10 mg doses of zolpidem (Cui et al., 2013; Villain, Chèze, Tracqui, Ludes, & Kintz, 2004). The same hair washing and analytical methods were used in these studies; however, significantly different zolpidem concentrations (1.8-9.8 pg/mg, and 135-554.6 pg/mg respectively) were detected in the proximal segments (0-2 cm). Kintz et al. (2014) referred to these concentrations as far higher than normally encountered in their daily experience, showing that 400 pg/mg or higher more likely corresponds to chronic drug use rather than the single 10 mg administered in this study, and a risk of a possible misinterpretation of the data. However, one should consider the ethnicity of the study participants (three French volunteers: Villain, Chèze, Tracqui, et al., 2004; and 20 Chinese volunteers: Cui et al., 2013) and other factors such as interindividual variations, such as metabolism, age, gender, incorporation rates and hair colour. Shen, Shi and Xiang (2013) identified that the Chinese Han population were poor metabolizers of zolpidem due to the reduced activity of the CYP2C19 enzyme, which suggest that genetic factors may affect results. However, the highest observed concentrations of zolpidem in blood was 321.5 ± 58.9 ng/mL, 2-3 hours postadministration of a single 10 mg dose of a zolpidem tartrate tablet. This was observably higher than Caucasian blood concentration of 167 ± 34 ng/mL after the same dose administration (Weinling et al., 2006). However, this still cannot account for the 100-fold increase of zolpidem concentration in hair observed between these studies.

However, Sato, Uematsu, Yamada and Nakashima (1993) were somewhat successful in observing a correlation of controlled dose administrations of chlorpromazine to patients with the detected concentrations in their hair, but this was shown to be dependent upon the melanin content in the hair as higher concentrations of chlorpromazine were detected in darker pigmented hair than with white hair. Additionally, Polettini et al. (2012) administered controlled doses of methamphetamine, identifying a within-subject correlation of dosage with detected concentrations, and that the detection increased with hair that had higher melanin content. These findings highlight the importance of hair pigmentation, and the role of melanin in the binding of drug molecules as discussed in Section 3.2.

One interesting possibility postulated by Pötsch, Skopp and Moeller (1997) was that the conjugation of drug functional groups by either hydrolysis or reduction, or oxidation might occur to a lesser extent within the hair follicle, leading to greater drug polarity. The authors showed that many metabolic enzymes are able to catalyse phase I and phase II reactions within the hair follicle itself but these are likely to be present in very small concentrations. Such compounds detected included NADPH-P450-reductase, P450-aromatase, P450-*O*-deethylase, along with alcohol dehydrogenase, carboxylases, esterase D, glutathione reductase, glycosyl-transferase, glutathione-*S*-epoxide transferase and sulfotransferase (Pötsch, Skopp, & Rippin, 1997). This area would benefit from more investigation as the presence of several cytochrome P450 isozymes are important for the metabolism and biotransformation of many drugs (Ogu & Maxa, 2000).

Overall, drug metabolism varies markedly between individuals, indicating that accurate dose-hair concentration determinations are unlikely. Such intervariation between individuals include hormone levels from the pancreas, pituitary, thyroid and adrenal glands, disease, age, gender and genetic variation. The cytochrome P450 2D6 for example has been shown to metabolize 25% of clinical drugs. It is also found in smaller quantities within Caucasian individuals than in other

TABLE 1 Stages leading to variability in hair analysis

STAGE 1: Drug-pharmacokinetic Interactions	
• Drug dosage administered • How the drug was administered • How often the drug was administered. • Pharmacokinetic parameters of the drug • Variation in metabolism between individuals	**Uncontrollable variables**

STAGE 2: Drug and Metabolite - Hair Interactions	
• Variable hair growth rates between individuals • Uncertainty of drug entry mechanism(s) into hair • Lipophilicity and permeability of drug-cell membrane • Drug/metabolite melanin content/pigmentation • Axial migration/drug distribution issues • Cosmetically treated/untreated hair • Quality of hair damaged/undamaged	**Uncontrollable variables**

STAGE 3: The Analyst and the Analytical Process	
• Sample collection and storage • Decontamination and wash/solvent choice • Analytical technique • Analysis and data interpretation	**Controllable variables**

races (Wang et al., 2009). Of course, lifestyle choice may also affect drug metabolism, for example, diet, vitamins, minerals and nutrients, health and smoking (Gibson & Skett, 2001; Taxak & Bharatam, 2014), while Kronstrand and Scott (2006) suggest that the physiochemical properties of a drug are more important than its plasma concentration.

3 | STAGE 2 VARIABLES: DRUG AND METABOLITE-HAIR INTERACTIONS

3.1 | Hair anatomy and rate of growth

The second stage of uncontrollable variables include the manner in which the drug and metabolites interact with and enter hair. This is an area still not understood, with knowledge advancing very little over the last 40 years and therefore it must be interpreted with caution. The hair itself may display its own variability that can affect data interpretation, for example varying rates of growth, axial migration of drugs along the hair shaft, and natural or cosmetic damage to the hair.

According to Harkey (1993) hair contains 65%-95% protein, 15%-35% water and 1%-9% lipids, which can vary between individuals depending upon factors that may include hair damage from heat, light, cosmetics and moisture content. The hair follicle and sebaceous glands are held within the surface of the skin in a pilosebaceous unit at the site of hair cell synthesis, hair growth, keratinization and melanin production. The cuticle, the outermost layer consists of overlapping dead keratinized cells approximately 0.5 μm thick. The cuticle, constructed of two layers, i.e., (a) the epicuticle outer layer (a lipoprotein membrane; Lyman & Schofield, 2008; Swift & Smith, 2001), and (b) a high cysteine containing inner cuticle (Yang, Zhang, & Rheinstädter, 2014). The cortex (the hair fibre core) resides beneath this (Yang et al., 2014) and is subdivided into three regions: (a) orthocortex; (b) paracortex; and (c) mesocortex. Melanin is found within the cortex, and is responsible for hair pigmentation (Harkey, 1993).

Cytokine activity is suspected to be an important contributor in the role of hair growth (Paus, 1998). Hair growth occurs through a looped three-stage cycle (Oh et al., 2016). Eighty-five per cent of hairs on the adult head are within the growth stage (anagen phase) at any one time, lasting between 4 and 8 years with a rate of between 0.22 and 0.52 mm per day (Kintz, 2016), or 1.06 cm per month (LeBeau et al., 2011). The mean growth rate has become accepted as 1 cm per month (Kintz, 2013; Pragst & Balikova, 2006); however, Pragst and Balikova (2006) state that a more accurate value would be 1.1 ± 0.2 cm/month. This is followed by a transition stage known as the catagen phase. Cell apoptosis occurs in a transition stage (catagen phase) following growth, lasting approximately 2 weeks, with up to one-third of hair cells being destroyed. This subsequently leads to an approximate 10-week resting stage (telogen phase), where hair growth remains dormant, before returning to the beginning of the cycle. Although an estimate, due to interperson variation, knowledge of the rate of hair growth is important in hair analysis, most notably for segmental hair analysis (see Section 4.3) where its growth rate allows for an approximate timeline of when the drug exposure took place. Harkey (1993) described the now accepted average growth rate of hair of 1 cm per month as an oversimplification as hair growth can be affected by variables that include age, gender and ethnicity. However, particular attention to interpretation should be applied to adolescent cases, as hair has been shown to grow at asynchronous rates (Kintz, 2017). It has been acknowledged that the rate of growth of hair can vary during the growth cycle and between hairs of the same region of the head (Kintz, Villain, Dumestre-Toulet, & Ludes, 2005; LeBeau et al., 2011). Studies have investigated growth rates of human hair with various results. Table 2 displays 15 studies between the years of 1951 and 2018, with a total average mean growth rate of 1.16 ± 0.27 cm/month and range of 0.86-1.98 cm/month.

3.2 | Drug incorporation routes into hair

The route of drug and metabolite entry into hair is not fully understood (Pragst & Balikova, 2006; Yu et al., 2017). Previous studies have contributed to many theories and these are summarized in Figure 1, yet knowledge has advanced very little during the last 40 years. Proposed routes for drug and metabolite entry have included passive diffusion directly from blood capillaries surrounding the follicle (Cone, 1996; Polettini et al., 2012), drug containing excretions from the sebaceous glands passing into hair during growth within the follicle (Harkey, 1993), the incorporation of the drug and metabolite into the hair follicle from sweat and sebum during growth (Harkey, 1993; Kintz, 2013) and external contamination from environmental exposure (Henderson, 1993; Tsanaclis & Wicks, 2007). Melanin is widely regarded as the main contributing factor for drug binding and incorporation, although its physiological function and binding mechanisms have not been proven (Pötsch, 1996; Testorf, Kronstrand, Svensson, Lundström, & Ahlner, 2001). Larsson and Tjälve (1979) proposed that negatively charged carboxyl groups, phenolic OH groups and semiquinones on melanin could be responsible for ionic drug-binding sites, with Van der Waals and hydrophobic interactions contributing to a minor extent, with a suggestion that positive radicals and basic drugs have higher affinities for binding to melanin (Larsson & Tjälve, 1979). Other studies identify that the quantity of melanin in hair does play an important role for how much drug is incorporated and thus detectable by hair analysis, with a correlation of increased drug incorporation observed for hair with higher melanin content (Polettini et al., 2012; Pragst & Balikova, 2006; Slawson, Wilkins, & Rollins, 1998). Darker hair and pigmented hair has generally been shown to have a higher melanin content, and as such, hair colour has been observed to play a crucial role for the effectiveness of drug detection (Kronstrand & Scott, 2006; Pötsch, Skopp, & Moeller, 1997; Pragst & Balikova, 2006; Yu et al., 2017). Sato et al. (1993) observed notable differences in the detected quantities of chlorpromazine in pigmented and nonpigmented hair using high-performance liquid chromatography after controlled doses were

TABLE 2 Human hair growth rates from previous studies

Minimum (cm/month)	Maximum (cm/month)	Mean (cm/month)	Reference
1.13	1.55	1.34	Kuwayama et al., 2018
1.20	1.40	1.30	Wang et al., 2016
0.50	1.50	1.00	Xiang, Shen, & Drummer, 2015
0.70	1.40	1.05	Kintz, 2013
Not known	Not known	1.09 ± 0.20	Scott, 2009
0.73	1.48	1.11	Tajima et al., 2007
0.84	1.41	1.13	Pragst, 2004
0.95	1.12	1.04	Van Neste, 2004
0.65	2.20	1.43	Pötsch, 1996
0.60	3.36	1.98	Harkey, 1993
0.90	1.20	1.05	Bost, 1993
0.84	1.37	1.11	Miyazawa & Uematsu, 1992
0.76	0.96	0.86	Pecoraro, Astore, Barman, & Araujo, 1964
Not known	Not known	0.94	Barman, Astore, & Pecoraro, 1964
0.84	1.15	1.00	Myers & Hamilton, 1951

administered to volunteers. Detection in nonpigmented hair was found to be <10% of the observed amounts in black hair. The authors described detection in nonpigmented hair as much lower and even undetectable. In contrast to these findings, Kelly, Mieczkowski, Sweeney and Bourland (2000) studied differential concentrations of amphetamine, cannabinoids and cocaine in hair of various pigmentations and races from 2000 samples. It was concluded that there was very little evidence to suggest that drug binding was dependent upon hair colour and race alone, and suggested that drug preferences between societal groups may provide a role in variation.

Investigations by Nakahara, Takahashi and Kikura (1995) identified that the rate of drug incorporation into hair depended highly upon a combination of melanin affinity and lipophilicity of the drug, and the pH gradient across the hair membrane, suggesting that hair of a lower pH, with more melanin will have a better affinity for the incorporation of basic drugs. Testorf et al. (2001) had demonstrated the characterization of flunitrazepam binding to cuttlefish melanin, but advised that it could be possible for flunitrazepam to be displaced from its melanin-binding site by drugs with a greater affinity to melanin. This could obviously lead to a problematic interpretation of data if a person was exposed to more than one drug at any one time, with the drug of greater affinity being detected in greater quantities than the drug of lower affinity.

A list of variables that may affect the uptake and movement of drug molecules across hair membranes have been reported. This includes lipid solubility of the drug, its molecular mass, ratio of ionized to nonionized drugs, pH gradient, blood flow and concentration gradient (Pötsch, Skopp, & Moeller, 1997). Chemical properties of drugs, which may allow them to be more likely incorporated into hair such as having a mass lower than 800 Da, a nitrogen atom for binding to melanin, non-acidic, lipophilic (allowing passage through cell membranes), N-alkyl chains, and an N-benzene ring etc. have been mentioned (Kintz, 2012a). All these factors are beyond the control of the analyst and therefore fall within the stage 2 variables shown in Table 1.

There has been recent progress made in terms of understanding drug incorporation routes into hair. Matrix-assisted laser desorption ionization-time of flight-imaging mass spectrometry (MALDI-TOF-IMS) of 30 mm longitudinally cut hair segments by Kamata et al. (2015) revealed that methoxyphenamine administered to volunteers (50 mg) had entered the hair by two routes, i.e., (a) hair bulb, and (b) keratinized upper dermis. Furthermore, Shima et al. (2019) observed that after a single dose of 50 mg methoxyphenamine and 10 mg zolpidem the incorporation of drugs through these two routes was dependent on both the lipophilicity of the drug, and the presence of hair pigmentation. Observations in this study showed that pigmented hair had incorporated both of these drugs through both the hair bulb and the keratinized upper dermis, while nonpigmented white hair had incorporated predominantly through the hair bulb. As suggested by the authors, these observations likely show that pigmentation (melanin) does not contribute an important role in the uptake of drugs being incorporated through the upper dermis by external contamination, sweat and sebum, but is important as part of the incorporation mechanism through the hair bulb. The study had also identified that the zolpidem, which had been incorporated into the hair bulb was less likely than methoxyphenamine to be further incorporated into the hair shaft during the hair growth phase, suggesting that this is likely attributed to the higher lipophilicity of zolpidem. This is also in agreement with other observed and expected drug lipophilicity behaviour (Kintz, 2012a; Nakahara et al., 1995; Shima et al., 2019).

Drug incorporation findings would obviously benefit by further investigation, and the different physiochemical properties between drug groups and hair studied. However, it may be just as likely that no specific drug incorporation model can be applied, requiring accurate hair dose-concentration studies to have specific models for each individual.

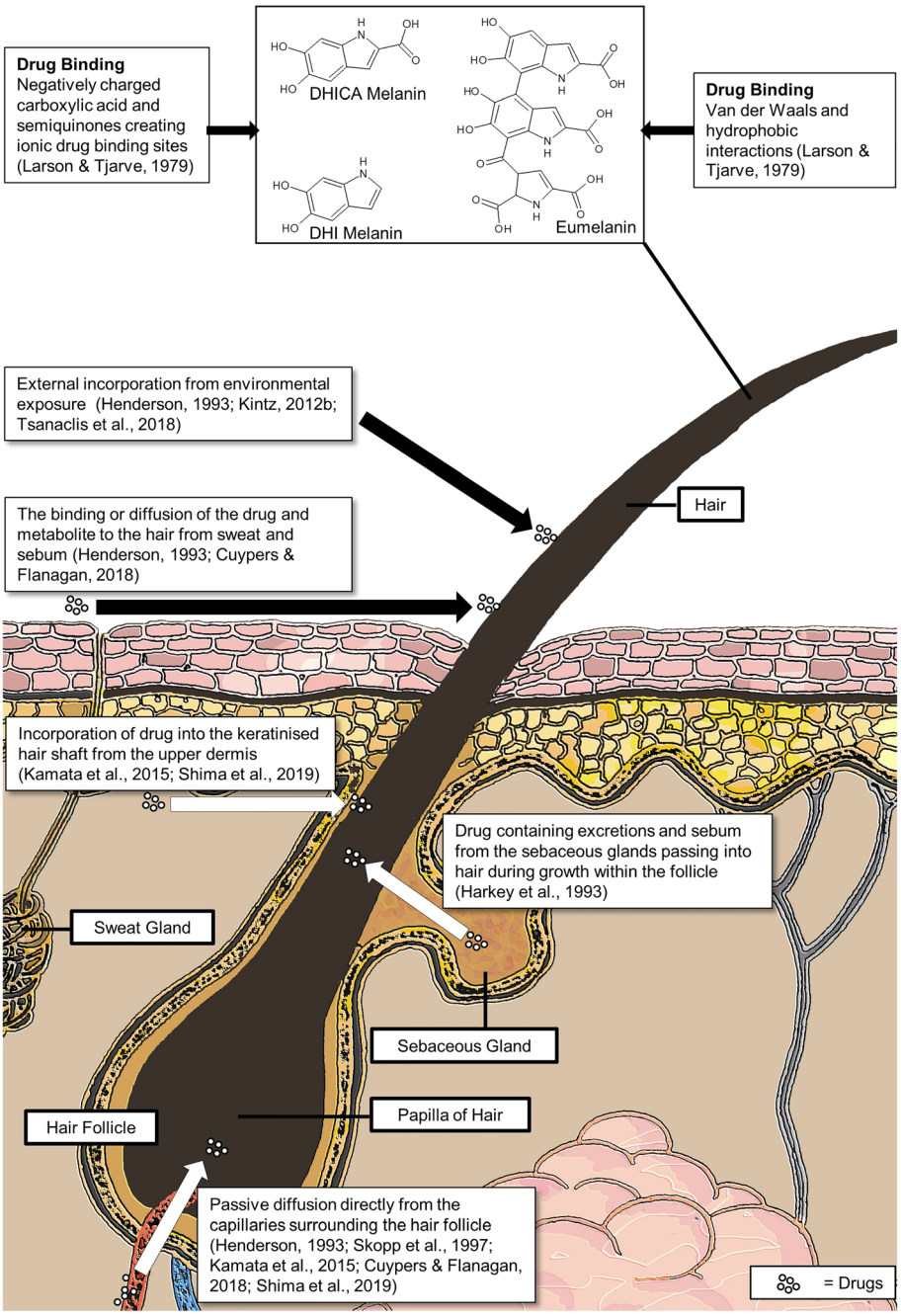

FIGURE 1 Proposed drug incorporation routes into hair

3.3 | External contamination and cosmetic damage

External factors such as contamination from the environment, and passive exposure leading to false positive interpretation can be avoided with appropriate application and interpretation of washing/decontamination procedures. For example, external drug contaminants coming into contact with the hair through passive means would display an observably higher concentration of drug in the wash solution than extracted from the hair itself (Pragst, 2004). The author also suggested the possibility of differentiating between external contamination by the correct interpretation of drug metabolite ratios. However, as discussed in Section 4.2, one must also consider the suitability of the wash solution, so as to minimize leaching of the drug from the hair itself. Another route of passive exposure in the case of children may occur from in utero exposure due to parental consumption, and it has also been noted that adolescent hair is more porous than adult hair leading to easier incorporation from external sources such as sweat (Kintz, Farrugia, Ameline, Eibel, & Raul, 2017). Cuypers et al. (2016) have demonstrated that certain wash procedures may promote external drug contaminants to enter the hair matrix (Section 4.2).

It is well known that the structure of hair can be damaged as a result of heat, light and cosmetics, which may allow for irregular movement and incorporation of drug along the hair shaft from sweat and sebum, or by causing swelling of the hair and increasing its water absorption capacity (Harkey, 1993; Kintz, 2013), which could be a

contributing factor to the variability of detected drug concentrations. It has been acknowledged that not only can cosmetic damage increase or decrease the detectable levels of drug, but also the manner that it is transported or incorporated along the hair shaft, leading to, for example, axial migration (see Section 3.4) (Kintz, 2013; Salomone et al., 2012; Wang et al., 2016).

The photosensitivity and effect of ultraviolet (UV) light on some drugs in hair should also be interpreted with care (Sato et al., 1993). The authors suggested that as hair is always exposed to light, photosensitive drugs that are prone to degradation in UV light should be taken into account. An example of this is shown within a study carried out by Favretto et al. (2013) who had investigated the photodegradation of methadone, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 6-monoacetylmorphine, morphine, cocaine and benzoylecgonine within positive hair samples when exposed to UVB radiation at 300 J/cm^2. The authors noted that the drugs degraded by an average of 55%, 17%, 21%, 17%, 20% and 11% respectively. Importantly, this indicated that methadone was highly susceptible to photodegradation in hair, with benzoylecgonine being the most stable of the selected drugs, with just 11% degradation. As with many other studies, the authors observed that hair pigmentation had diversified the results, with greater photodegradation of methadone and EDDP occurring within lighter coloured hair than in darker hair. The authors suspected that the higher eumelanin content in darker hair had aided with the protection of these drugs, but also speculated that melanin may also undergo photodegradation when exposed to superoxide anions caused by exposure to UV light and oxygen.

The effect that bleaching and dyeing of hair has on the detectability of cocaine, opiates, cannabinoids and nicotine has been studied (Jurado, Kintz, Menéndez, & Repetto, 1997). Volunteers with bleached and dyed hair, who had a history of drug abuse, were selected. Strands of hair were segmented to separate the bleached or dyed portions with untreated sections before extraction and analysis by gas chromatography-MS. The authors observed an overall mean drug reduction of 40%-60% between all treated and untreated hair, and lower concentrations of drug detected in bleached hair than in dyed hair. Therefore, factors such as cosmetic treatments, environmental and external contamination should be considered when interpreting hair analysis results.

3.4 | Concentration variation associated with axial migration and age

The implementation of segmental hair analysis is without doubt a useful technique. However, it does have limitations, particularly estimating the timeframe of drug exposure. The issue of axial migration of a drug along the hair shaft has been described and addressed (Kintz, 2013). Axial migration can be problematic for segmental analysis if not interpreted correctly, most importantly when corroborating the timeframe of an alleged drug-facilitated incident. The author acknowledged that the detection of a drug in consecutive hair segments was observed within approximately 10% of cases (1 in every 10 cases), signifying that accurate drug consumption timeframes could be misinterpreted. It is still not known why irregular movement of a drug occurs along the hair shaft, although a variety of influencing factors should be considered, for example, changes in the hair structure, cosmetic treatments and hair damage. One such cause of external route of entry into the hair has been suggested to originate from the irregular diffusion of drugs from sweat and sebum while the hair grows (Wang et al., 2016). Therefore, it has been proposed that for a single drug exposure, the segment with the highest concentration should represent the time of an alleged incident, providing that it is at least three times higher than the surrounding segment concentrations (Kintz, 2013).

Analysis of hair from children has been shown to be particularly problematic in determining single or prolonged repeated drug exposure. Kintz et al. (2017) demonstrated that methadone detection in the hair of four children was approximately the same in each consecutive hair segment for each individual, likely due to absorption from sweat. Alvarez et al. (2017) recently reported that the distribution of drugs along the hair shaft was extensive among children under the age of 36 months yet reduces after this age. It was proposed here that due to the hair of children being much thinner and more porous than adult hair, it would be more prone to absorption of a drug from sweat and sebum.

Salomone et al. (2012) identified the presence of zolpidem in three hair segments of a DFSA case. Two of the segments were consecutive (2-3, 5-6 and 6-8 cm). These findings suggest consumption on more than one occasion. However, the authors also proposed other considerations: (a) thermal or cosmetic damage of the hair; (b) swelling of the hair and an increase in water absorption capacity due to damage, aiding the migration of drug along the hair shaft; and (c) incorporation of the drug during the growth cycles of hair.

4 | STAGE 3 VARIABLES: THE ANALYST AND THE METHOD

4.1 | Variation associated with hair sample collection and processing

Stage 3 contains variables that are within the control of the analyst. Of major importance is the selection of a suitable methodology and the use of appropriate analytical instrumentation. With respect to initial hair cutting and collection, there is no standardized method and different techniques have been applied. The Society of Hair Testing (SoHT) (Cooper, Kronstrand, & Kintz, 2012) have a recommended method of hair collection, and as such, it has become a fundamental and accepted step for the analysis of hair. Hair samples should be taken at a minimum of 4-6 weeks after the alleged incident, and ideally cut from the posterior vertex area of the head. A lock of hair of pencil thickness should be cut as close to the scalp as possible using scissors, so that the first segment correlates to the previous month's growth phase. The cut root hair end should be clearly identified by

wrapping foil around it, and each sample should have its own individual collection envelope sealed with tape, placed into evidence collection bags and stored in a dry environment at room temperature out of direct sunlight. Hair colour and any hair treatments before cutting should be noted and written on the collection envelope.

LeBeau et al. (2011) studied the accuracy of hair collection and cutting techniques of 14 individuals, so as to determine how close to the scalp the cuts were made. An observed variation of 0.8 ± 0.1 cm of hair was left on the scalp after cutting. Therefore, the authors recommend a period of 8 weeks after ingesting a possible drug before collecting hair specimens, particularly when segmental analysis is required, stating that inconsistency with collection methods and the variability of hair growth rates can significantly affect the interpretation of each analysis.

SoHT methodology (Cooper et al., 2012) states that the posterior vertex region of the head displays the least growth rate variability, and was confirmed by Polettini et al. (2012) in a study that collected hair samples from multiple regions of the head, noting that higher concentrations of detectable amphetamine and methamphetamine were observed within the posterior vertex, temporal vertex and the nape, than detected in the frontal and anterior vertex regions of the head.

4.2 | Decontamination/washing considerations

Consideration of what the best washing and decontaminating method is has been described by Tsanaclis, Andraus and Wicks (2018) as an issue that arouses controversy in hair analysis. However, the choice is a crucial step for eliminating impurities and analytical interferences. The SoHT guideline outlines several purposes of hair washing that include: (a) removal of hair care products; (b) sweat, sebum and skin cells; (c) head lice; and (d) other body fluids. Any decontaminating procedures must remove any external contamination without extracting the internal drugs and analytes in question. Tsanaclis and Wicks (2007) recommended that a hair washing procedure should be both practical and should be able to remove drugs from the external surface of the hair. It should also be noted there is no agreement upon any technique for identifying external contamination (Kintz, 2012b).

Wash solvents can be of two types, i.e., protic and aprotic. Protic solvents such as methanol and other aqueous solutions should be applied and interpreted carefully, as they can swell the hair, leading to the extraction of analytes during the wash stage (Cooper et al., 2012). Methanol for example, will not only remove soluble contaminants from the surface of hair, but can also extract drugs/analytes. In a study conducted by Tsanaclis and Wicks (2007) they had included a 1-minute methanol wash to limit this extraction process. Nonprotic solvents are described as more reliable, as they do not swell the hair. However, the SoHT guidelines recommend washing stages that include both organic solvents and aqueous solutions (Cooper et al., 2012). Presently, there is no standard hair washing method, and no agreement on what the most effective hair washing procedure is. Studies have applied, experimented and built upon previous techniques, and it is apparent that there is a preferred order of decontaminating/washing before segmenting/cutting to reduce leaching of the analyte from the hair into the wash material.

Previous researchers have also applied and investigated many forms of decontamination and washing methods (Table 3). The processes range from applying a single wash solvent (Aleksa et al., 2012; Cirimele et al., 1997), to multiple wash solutions (Harun et al., 2010; Kłys et al., 2007; Miller et al., 2006), as well as wash solvents and solutions that have included soap/detergent (Baumgartner et al., 1979; Fernández et al., 2009; Míguez-Framil et al., 2011), ethanol (Kidwell, Lee, & DeLauder, 2000), acetone and hexane (Licata et al., 2016), sodium dodecyl sulphate (Favretto et al., 2011; Nakahara & Hanajiri, 2000) and isopropanol (Montesano et al., 2014).

Dichloromethane washes with or without ultrasonification appear to be the most common wash procedures used (Anderson et al., 2008; Fisichella et al., 2014; Jurado et al., 1997; Maublanc et al., 2014). Duvivier, Peeters, van Beek and Nielen (2016) investigated solvent-based washing methods for decontamination of Δ9-tetrahydrocannabinol (THC) in contaminated hair samples. All three methods studied performed equally well at removing THC contamination from hair, these methods were: (a) methanol followed by sodium dodecyl sulphate (SDS) wash; (b) methanol, SDS followed by methanol wash; and (c) two times methanol wash. The authors advised that a two-step wash procedure consisting of methanol followed by aqueous SDS solution, in line with the SoHT guidelines (Cooper et al., 2012) would be sufficient. Previously, Paulsen, Wilkins, Slawson, Shaw and Rollins (2001) had evaluated four different hair decontamination methods for the detection of cocaine and its metabolites in rat hair. These methods were: (a) methanol; (b) phosphate buffer at pH 6; (c) phosphate buffer at pH 8; (d) isopropanol followed by phosphate buffer at pH 8; and (e) no wash at all. The study showed that each wash method varied in efficiency, and had removed varied quantities of incorporated cocaine and its metabolites from within the hair, with no single wash method being optimum across the range of drugs. Importantly, this study showed that the choice of wash procedure could likely contribute to the variability in quantity of drugs detected between laboratories; therefore, the authors recommend that any decontamination procedure chosen should be validated for its effectiveness when interpreting results.

More recently, the application of MALDI-TOF-IMS and metal assisted-secondary ion MS (MetA-SIMS) techniques were utilized by Cuypers and Flanagan (2018) to compare the spatial distribution of hair externally spiked with cocaine with hair obtained from cocaine users. The authors identified two important factors regarding the reliability of the wash solvents dichloromethane and acetone, i.e., (a) these solvents did not remove all of the cocaine surface contaminants, and (b) MetA-SIMS analysis revealed that the solvents might have promoted the movement of contaminants from the surface of the hair, and into the hair itself. The authors did acknowledge that their contaminated samples would be of a higher concentration than expected in real life samples, but rightfully pointed out that their technique does show that the movement of contaminants into the

TABLE 3 Summary of methods used in drugs detection from hair

Washing/decontamination	Buffer solutions	Extraction	Analysis	Analyte(s)	Reference
Detergent ×3, water ×3	Phosphate buffer pH 7.4	Pulverized, methanol (heated for 2 h), centrifugation, evaporation	RIA[7]	Heroin and MOR[25] metabolites	Baumgartner, Jones, Baumgartner, & Black, 1979
Not described	Tris buffer pH 7.1	Toxi-tube A digested with pronase and dithiothreitol	GC-MS[8]	COC[24], BZE[26], EME[27], MOR[25], COD[28], 6-MAM[29],	Barrera & Rossi, 1995
Dichloromethane ×2 (2 min)	Sorensen phosphate buffer pH 7.6 (40°C for 2 h)	Diethylether/chloroform (80:20, v/v), agitated (20 min), centrifugation (15 min)	GC-MS-NCI[9]	Flunitrazepam	Cirimele, Kintz, & Mangin, 1996
Dichloromethane ×2 (2 min)	Sorensen phosphate buffer pH 7.6 (40°C for 2 h)	Diethylether/chloroform (80:20, v/v), agitated, centrifugation (15 min)	GC-MS-NCI[9]	Flunitrazepam, 7-aminoflunitrazepam	Cirimele, Kintz, Staub, & Mangin, 1997
Ethanol/methanol (9:1, v/v) (3 min)	With and without acetate buffer pH 4	Multiple: (a) methanol; (b) arylsulphatase/ β-glucuronidase SPE[1];(c) aqueous urea solution SPE[1]; (d) acetone; (e) 2-propanol	GC-MS[8]	COC[24], BZE[26], MOR[25], MAM[31]	Eser, Pötsch, Skopp, & Moeller, 1997
Dichloromethane ×2 (15 min)	None	HCl[21], organic solvent	GC-MS[8]	MOR[25], BZE[26], COD[28], 6-MAM[29], THC[32], THC-COOH[33], nicotine	Jurado et al., 1997
Sodium dodecyl sulphate ×3, water ×3 (rinsed)	None	Methanol/HCl[21] (20:1, v/v, sonification for 1 h), trifluoroacetic anhydride/ethyl acetate (1:1, v/v, 60°C for 20 min)	GC-MS[8]	7× MAMPs[35]	Nakahara & Hanajiri, 2000
Methanol (1 h sonification)	None	Methanol (sonification 1 h), centrifugation (5 min), HCl[21] (vortexed 55°C overnight), acetic acid and water, SPE[1]	GC-MS[8]	Clonazepam, 7-aminoclonazepam	Negrusz et al., 2000
Methanol (sonification 1 h + centrifugation for 5 min)	None	Methanol, water, acetic acid, HCl[21], (dichloromethane, isopropanol, ammonium hydroxide 78:20:2, v/v), SPE[1]	GC-MS[8]	7-aminoclonazepam, clonazepam	Negrusz et al., 2002
Sodium dodecyl sulphate and water ×3	Multiple compared	Multiple extraction methods compared: (a) proteinase K; (b) basic methanolic; (c) acidic methanolic; (d) Soerensens buffer; (e) NaOH[22]; (f) β-glucuronidase; (g) protease	HPLC[10] and GC-MS[8]	BZPs[36]	Scott & Nakahara, 2003
Dichloromethane ×2 (5 min)	Sorensen buffer pH 7.6 (56°C for 14 h)	Dichloromethane, LLE[2] (10 min), centrifugation	LC-MS/MS[11]	Bromazepam, clonazepam, metabolites	Chèze et al., 2004

(Continues)

TABLE 3 (Continued)

Washing/decontamination	Buffer solutions	Extraction	Analysis	Analyte(s)	Reference
Dichloromethane	Phosphate buffer pH 8.4 (overnight)	Dichloromethane/diethyl ether (80:20, v/v), centrifugation (15 min)	LC-MS/MS[11]	Lorazepam	Kintz, Villain, Cirimele, Pépin, & Ludes, 2004
Dichloromethane	Phosphate buffer pH 8.4 (overnight)	Dichloromethane/diethyl ether (90:10, v/v), agitation and centrifugation (15 min)	LC-MS/MS[11]	Tetrazepam	Concheiro et al., 2005
Dichloromethane ×2 (2 min)	Phosphate buffer pH 8.4 (overnight)	Dichloromethane/diethyl ether (80:20, v/v)	LC-MS/MS[11]	Alprazolam	Kintz, Villain, Chèze, & Pépin, 2005
Dichloromethane ×2 (2 min)	Phosphate buffer pH 8.4 (overnight)	Dichloromethane/diethyl ether (90:10, v/v), agitation (15 min), centrifugation (15 min)	LC-MS/MS[11]	16× BZPs[36], hypnotics	Villain, Concheiro, Cirimele, & Kintz, 2005
Aqueous sodium dodecyl sulphate ×1 (10 min ultrasonification), deionized water ×2 (10 min ultrasonification), dichloromethane ×2 (10 min ultrasonification)	Phosphate buffer pH 6.0	Methanol/NaOH[22] (20:1, v/v, sonification for 1 h, then left overnight), second wash performed with methanol	ELISA[13] and LC-ESI-MS/MS[12]	BZPs[36]	Miller, Wylie, & Oliver, 2006
Not explained	Ammonium chloride pH 9.2	Methanol (45°C shaking for 2 h), 1-chlorobutane, formic acid, water/methanol (70:30, v/v)	LC-MS/MS[11]	26× BZPs[36]	Laloup et al., 2007
Water and acetone (×2)	None	Methanolic HCl[21] (50 °C ultrasonification for 1 h)	GC-MS[8]	Phenylalkylamine derivatives	Kim, Jung, Kim, Lee, & In, 2007
n-hexane (vortexed 30 s), acetone (vortexed (30 s)	None	Methanol (vortexed and ultrasonification at 50°C for 1 h, left overnight)	LC-APCI-MS/MS[14]	OPIs[37], AMPs[38], COCs[24]	Kłys, Rojek, Kulikowska, Bożek, & Ścisłowski, 2007
Shampoo, water, dichloromethane, isopropanol, acetone	Phosphate buffer pH 7.0 and potassium hydroxide pH 7.0	HCl[21] (50°C for 16 h), SPE[1] (methanol, water and phosphate buffer pH 7.0)	GC-MS[8]	OPIs[37], AMPs[38], COC[24], diazepam, metabolites	Cordero & Paterson, 2007
Aqueous sodium dodecyl sulphate ×1 (10 min ultrasonification), deionized water ×2 (10 min ultrasonification), dichloromethane ×2 (10 min ultrasonification).	Phosphate buffer pH 6.0 for SPE[1], toluene for MISPE[4]	Methanol/ammonium hydroxide (20:1, v/v), ultrasonification (1 h, left overnight), second wash of methanol/ammonium hydroxide (20:1, v/v), SPE[1] and MISPE[3] compared	LC-MS/MS[11]	BZPs[36]	Anderson, Ariffin, Cormack, & Miller, 2008
Soap ×3 (10 min), water ×3 (rinsed)	None	Microwave assisted extraction (methanol, 60°C for 9 min)	HPLC[10]	OPIs[37], COC[24] and metabolites	Fernández et al., 2009
Dichloromethane ×3	Formic acid pH 4.0	Methanol, ultrasonification (40°C for 4 h)	LC-MS/MS[11]	MOR[25], COD[28], 6-AM[30], BZE[26], COC[24]	Huang, Liu, Huang, & Chien, 2009

(Continues)

TABLE 3 (Continued)

Washing/decontamination	Buffer solutions	Extraction	Analysis	Analyte(s)	Reference
Water (shaking 4 min), acetone (shaking 4 min), petroleum ether (shaking 4 min)	None	Extracted twice: alkaline hydrolysis then LLE[2], and methanolic extraction	GC-MS[8]	Δ9-THCA[34], THC[32]	Auwärter, Wohlfarth, Traber, Thieme, & Weinmann, 2010
Shampoo, sodium dodecyl sulphate, water, dichloromethane	Phosphate buffer pH 5.0 (45°C for 18 h)	Methanol/NaOH[22], MISPE[3] (acetonitrile and phosphate buffer pH 5.0, vortexed, eluted in 30% acetic acid in acetonitrile)	LC-MS/MS[11]	KET[39], norKET[40]	Harun, Anderson, & Cormack, 2010
Methanol ×3	Phosphate buffer pH 7.0	HCl[21] (37°C for 16 h), vortexed and centrifugation (5 min), SPE[1]	GCxGC-TOF-MS[15]	Multiple	Guthery, Bassindale, Bassindale, Pillinger, & Morgan, 2010
Methanol ×4	Acetonitrile pH 5.3	Methanol/acetonitrile/ammonium formate (25:25:50, v/v/v), centrifugation (10 min), incubation (37°C for 18 h), centrifugation (10 min)	UPLC-TOF-MS[16]	52× pharmaceutical, and drugs of abuse	Nielsen, Johansen, Dalsgaard, & Linnet, 2010
Sodium dodecyl sulphate ×1 (3 min), water ×2 (3 min), acetone ×1 (3 min)	Phosphate buffer pH 4.0	Methanol/TFA (90:10, v/v), ultrasonification (1 h), 45°C overnight, dichloromethane/diethyl ether LLE[2]	HPLC-HRMS[17]	AMPs[38], COC[24], OPIs[37], BZPs[36], antidepressants, hallucinogens	Favretto et al., 2011
Methanol, water/methanol ×2	None	Methanol (38°C for 16 h)	LC-MS/MS[11]	27× BZPs[36] and zolpidem	Kim, Lee, In, Choi, & Chung, 2011
Water ×1, methanol ×1	Phosphate buffer pH 6.0	Methanol (38°C for 16 h), SPE[1]	GC-MS[8]	Hypnotics	Lee et al., 2011
Dichloromethane ×3 (2 min)	Borate buffer pH 9.0	Acetonitrile (50 °C for 12 h), centrifugation (10 min at 4°C), then two-step extraction; LLE[2] and SPE[1]	LC-MS/MS[11]	Multiple, 35 drugs and metabolites	Lendoiro et al., 2011
Soap solution, water (stirred for 30 min)	Sodium borate 2 M/boric acid 2 M (50:50, v/v)	MSPD[4], SPE[1]	ESI-MS/MS[18]	OPIs[37], COC[24]	Míguez-Framil et al., 2011
0.5% Sodium dodecyl sulphate ×1Distilled water ×3 (ultrasonification 1 min) Methanol ×3 (ultrasonification 1 min)	Not required	Not required	MALDI-TOF-IMS, and MALDI-FTICR-IMS	MAMP[35]	Miki et al., 2011
Dichloromethane ×2 (vortexed 3 min)	Formic acid buffer	Methanol (55°C for 15 h)	LC-MS/MS[11]	Zolpidem and multiple	Salomone et al., 2012
Dichloromethane ×2 (rinsed)	Phosphate buffer pH 8.4 (ultrasonification 1 h)	LLE[2] (dichloromethane)	LC-MS/MS[11]	18× BZPs[36]	Xiang et al., 2011

(Continues)

TABLE 3 (Continued)

Washing/decontamination	Buffer solutions	Extraction	Analysis	Analyte(s)	Reference
Dichloromethane ×2 (1 min)	Phosphate-buffered saline pH 7.4	Methanol (agitation, 56°C for 18 h), HS-SPME[5]	GC-MS[8]	Multiple, 17 drugs and metabolites	Aleksa et al., 2012
Dichloromethane ×2 (vortexed 3 min)	None	Methanol (55°C for 15 h)	UHPLC-MS/MS[19]	Multiple	Di Corcia, D'Urso, Gerace, Salomone, & Vincenti, 2012
2-propanol (ultrasonification, 5 min), aqueous solution ×3 (ultrasonification 5 min) (washed after segmentation)	None	Centrifugation (20°C for 10 min), incubated (37°C for 18 h), centrifugation (10 min)	UPLC-MS/MS[20]	Triazolam and metabolites	Johansen & Dahl-Sørensen, 2011
Dichloromethane (vortexed 30 s), methanol (vortexed 30 s)	None	HCl[21] (brief vortex and centrifugation), incubated (45°C overnight), centrifugation (1 min), water (centrifugation 10 min)	LC-MS/MS[11]	COC[24] and metabolites	Alves et al., 2013
Methanol, water/methanol ×2	None	Methanol (38°C for 16 h), SPE[1]	LC-MS/MS[11]	BZP[36] and zolpidem	Kim et al., 2013
Dichloromethane ×2 (2 min)	Ammonium chloride buffer pH 9.5 (40°C overnight)	Incubated (45°C overnight w/buffer), LLE[2] [dichloromethane/ isopropanol/n-heptane (25:10:65, v/v)]	LC-MS/MS[11]	Amitriptyline, temazepam, tramadol, dihydrocodeine	Chatterton & Kintz, 2014
Dichloromethane, methanol	None	Methanol, ultrasonification (1 h)	LC-MS/MS[11]	87× psychoactive drugs	Fisichella, Morini, Sempio, & Groppi, 2014
Water ×2 (2 min), dichloromethane ×2 (1min)	Phosphate buffer pH 5 (shaking for 16 h)	NaOH[22], dichloromethane/ether (70:30, v/v), shaken (15 min), centrifugation (5 min), reconstituted with formate buffer (pH 3)/acetonitrile (90:10, v/v)	LC-ESI-MS/MS[12]	BZPs[36], hypnotics, psychotropics	Maublanc, Dulaurent, Morichon, Lachâtre, & Gaulier, 2014
Isopropanol ×1, water ×2 (rinsed)	Ammonium formate pH 5.3	Methanol/acetonitrile/ammonium formate (25:25:50, v/v), centrifugation (3 min at 20°C), incubated (37°C for 18 h)	UPLC-MS/MS[20]	96× drugs (multiple classes)	Montesano, Johansen, & Nielsen, 2014
Methanol ×2, water ×2, methanol ×2	None	Methanol (stirring 38°C for 16 h)	LC-MS/MS[11]	NNEI[41], MAM-2201[31]	Saito, Sasaki, Namera, Kurihara, & Inokuchi, 2014
Wiped with tissue paper moistened with distilled water	Acetate pH 5.0	Pulverized with acetate buffer (5 min), ultrasonification (30 min), LLE[2] with chloroform-isopropanol (3:1, v/v), organic layer separated.	LC-MS/MS	MOP[47]	Kamata et al., 2015

(Continues)

TABLE 3 (Continued)

Washing/decontamination	Buffer solutions	Extraction	Analysis	Analyte(s)	Reference
Wiped with tissue paper moistened with distilled water	Not required	Not required	MALDI-TOF-IMS	MOP[47]	Kamata et al., 2015
Methanol ×2, water ×2, methanol ×2	None	Methanolic extraction (stirring 38°C for 16 h)	UHPLC-MS/MS[19]	Multiple	Kim et al., 2015
Water (2 min), acetone (2 min)	Ammonium acetate pH 4.0	Methanol (sonification 50-60°C for 4 h), formic acid	LC-MS/MS[11]	MDMA[42], MDA[43]	Madry et al., 2015
Dichloromethane, water	Phosphate buffer	Methanol (sonification 45°C for 2 h), LLE[2] (1-chlorobutane)	UHPLC-MS/MS[19]	BZPs[36] and Z-Drugs	Ramírez Fernández et al., 2015
Acetone ×2, hexane ×2	None	Methanol (45°C overnight), roQdSPE QuECheERS[6] sorbent kit	LC-MS/MS[11]	50× psychoactive drugs and metabolites	Licata et al., 2016
Water ×1 (1 min), acetone ×1 (1 min)	Ammonium formate pH 5.3	Methanol/acetonitrile/ammonium formate (25:35:50, v/v), centrifugation	LC-MS/MS[11]	EME[27], MOR[25], COD[28], AMP[38], 6-MAM[29], MAMP[35], MDMA[42], BZE[26], MDEA[44], COC[24], EDDP[45], MTD[46]	Cappelle et al., 2017
None described	None described	Ammonium acetate w/0.5% formic acid/MeCN[23] (3:1, v/v), sonification (10 min), room temperature (24 h)	LC-MS/MS[11]	Diphenhydramine	Kuwayama et al., 2018

Extraction:
[1]Solid-phase extraction.
[2]Liquid-liquid extraction.
[3]Molecularly imprinted solid-phase extraction.
[4]Matrix solid-phase dispersion
[5]Headspace solid-phase microextraction.
[6]Quick Easy Cheap Effective Rugged and Safe.
Instrumentation:
[7]Radioimmunoassay.
[8]Gas chromatography-mass spectrometry.
[9]Gas chromatography-mass spectrometry-negative chemical ionization.
[10]High-pressure liquid chromatography.
[11]Liquid chromatography-tandem mass spectrometry,
[12]Liquid chromatography-electrospray ionization-tandem mass spectrometry.
[13]Enzyme-linked immunosorbent assay.
[14]Liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry.
[15]Two-dimensional gas chromatography-time of flight-mass spectrometry.
[16]Ultraperformance liquid chromatography-time of flight-mass spectrometry.
[17]High-performance liquid chromatography-high resolution mass spectrometry.
[18]Electrospray ionization-tandem mass spectrometry.
[19]Ultrahigh-performance liquid chromatography-tandem mass spectrometry.
[20]Ultraperformance liquid chromatography-tandem mass spectrometry.

Compounds:

[21]Hydrochloric acid.
[22]Sodium hydroxide.
[23]Acetonitrile.
[24]Cocaine.
[25]Morphine.
[26]Benzoylecgonine.
[27]Ecgonine methyl ester.
[28]Codeine.
[29]6-Monoacetylmorphine.
[30]6-acetylmorphine.
[31]Monoacetylmorphine.
[32]Δ9-tetrahydrocannabinol.
[33]11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid.
[34]Δ9-tetrahydrocannabinolic acid A.
[35]Methamphetamine.
[36]Benzodiazepines.
[37]Opiates.
[38]Aphetamine.
[39]Ketamine.
[40]Nor-ketamine.
[41]N-1-naphthalenyl-1-pentyl-^{1}H-indole-3-carboxamide.
[42]Methylenedioxymethamphetamine.
[43]Methylenedioxyamphetamine.
[44]Methylenedioxyethylamphetamine.
[45]Ethylenedimethyldiphenylpyrrolidine.
[46]Methadone.
[47]Methoxyphenamine.

hair is possible when decontaminating. A list of published wash methods, buffer solutions and extractions are displayed in Table 3.

Recently, many detox shampoos are available commercially with claims of removing drug traces from hair. One such shampoo, Zydot Ultra Clean was investigated by Röhrich, Zörntlein, Pötsch, Skopp and Becker (2000). The highest concentration decrease from spiked hair was observed for amphetamine (−41.0%) followed by THC (−36.3%). No change was reported for 3,4-methylenedioxymethamphetamine or MDMA and −4.8% decrease was noted for cocaine. Therefore, analysts must consider the use of shampoo type while interpreting drug hair results.

4.3 | Segmental hair analysis

Segmental hair analysis is a useful tool for determining the longer history of drug use in an individual (Kintz et al., 2017), and for estimating and corroborating the timeframes of an alleged single drug exposure. The basic principles of segmental hair analysis rely on two assumptions, i.e., (a) drugs and their metabolites are incorporated into the hair shaft while it grows, and (b) rate of hair growth is approximately 1 cm per month for every individual person. Cutting the hair into segments of 1 cm and detecting in that segment would therefore give an approximate 1-month window for drug exposure. As a guide, hair samples should be collected between 4 and 6 weeks after an alleged DFC. Detection of a drug in only the proximal segment would corroborate a single exposure within the time period associated with that segment (Cooper et al., 2012). As discussed in sections 3.1 to 4.3, the accuracy of predicting a timeframe will depend upon several factors that include the variation in hair growth rates, how close to the scalp that the hair was cut, accuracy of the segmentation cutting process, axial migration and age of the individual to whom the hair belongs. Owing to these variations, the estimated timeframes are limited to between 1 and 2 months, depending upon the size of the segment. The SoHT guidelines recommend segments of between 10 and 30 mm for a detailed history of drug exposure, with smaller segments providing a more accurate representation for identifying single drug exposures (Cooper et al., 2012).

Care must be taken when interpreting data from segmental analysis, as demonstrated by Wang et al. (2016). Segmental hair analysis was applied after controlled single doses of 10 mg of diazepam administered to eight volunteers. Results indicated that 1 month after exposure, diazepam and nordiazepam was detected as expected in the highest quantity in the 0-2 cm proximal hair segments corresponding to the time of exposure; however, diazepam and metabolites were also detected in the 2-4-cm segment for all volunteers, and the 4-6-cm segments for two of the volunteers. Whether this is due to axial migration, or incorporation from sweat and sebum on the scalp during hair growth is still not understood. The authors do recommend that analysis should therefore be interpreted carefully, and that sample collection should be within 6 months of exposure, as they had also observed a decrease in detection when sampling after 10 months.

Many case studies have had success with corroborating the timeframe of an incident (Johansen & Dahl-Sørensen, 2011; Villain, Chèze, Dumestre, et al., 2004). For example, Johansen and Dahl-Sørensen (2011) described the successful use of segmental hair analysis within an alleged DFSA case where blood and urine sampling 20 hours after an alleged incident had proven negative. The victim's hair was collected 34 days after an alleged incident. Three × 2-cm segments were analysed by ultraperformance liquid chromatography (UPLC)-TOF-MS, and 1 pg/mg triazolam was detected in the segment corresponding to the alleged incident timeframe. This case not only demonstrates that it is possible to corroborate a timeframe of an alleged incident, but also displays the potential for identifying drugs that have proven to have short detectable half-lives (e.g., 1.5-5.5 hours for triazolam), which may have gone undetected from the blood and urine samples.

Further to these observations, MALDI-IMS and time-course MSI of two possible methoxyphenamine incorporation routes into hair (hair bulb and keratinized upper dermis) indicated that the potential for an accurate chronological history of drug exposure using segmental analysis may only be effective down to a window of 11 days due to the two separate entry routes (Kamata et al., 2015).

Smaller hair segmentations of ≤1 mm have recently been investigated (Kuwayama et al., 2018; Shima et al., 2016). Notably, Kuwayama et al. (2018) developed a microsegmental hair analysis method for determining the day of drug ingestion (diphenhydramine), using three volunteers. Diphenhydramine and its metabolite desmethyldiphenhydramine were detected in specific segments correlating with the days of consumption (±0.9 days). The authors did acknowledge that the variability of hair growth rates could introduce decreased reproducibility using this method. Clearly, this method displays potential that requires further studies on a larger scale; however, it does require the use of internal temporal markers (chlorpheniramine) for estimating the day of drug intake. The authors also state that this method does not cost any more to perform than standard techniques; however, it would likely extend the overall analysis time, due to the cutting of 0.4-mm segments, and increase the quantities of segments that require analysis. Nevertheless, this procedure clearly shows promise regarding researching time and drug distribution patterns in hair, but whether the technique can be further developed for real life case samples is questionable due to ethical considerations of consuming temporal markers. While, hair growth rates have been shown to vary on an interindividual basis, reducing estimated timeframes of an alleged drug intake from a month to a week and even a few days would be highly advantageous in criminal investigations.

4.4 | Extraction and analysis

The procedure used for digestion or extraction of the analyte(s) from the hair should be sufficient to extract analytes from the hair matrix and into solution, making it detectable by the chosen analytical instrument. Molecularly pregnated polymer solid phase extraction (MISPE), SPE (Anderson et al., 2008), liquid-liquid extraction, methanolic and

acidic hydrolysis (Cirimele et al., 1996), and micro-pulverized extraction (Favretto et al., 2011) have all been applied with varied success depending on the target analyte. The efficiency of the extraction seems somewhat dependent upon the type of analyte(s) under investigation, which provides difficulty in standardizing a method for simultaneous detection. In the case of benzodiazepines, MISPE provided a greater number of detections over SPE with regards to diazepam due to its lower limit of detection, whereas temazepam was detected more often with SPE (Anderson et al., 2008), and in another study, acid hydrolysis of hair containing cocaine proved more effective than methanolic and alkaline extraction (Cirimele et al., 1996).

Recent advances in analytical techniques have enabled the development of more sensitive methods for the detection of a single drug exposure, whereas previously, only chronic use was detectable in hair. Choosing an appropriate analytical method and the use of validated parameters is essential. Kintz (2012a) acknowledged that a negative test result for a drug in hair does not necessarily indicate that the drug was not present, as some hair analysis cases have provided negative results when blood and urine tests were shown to be positive, and suggested that in which case the sensitivity of the hair testing method should be questioned and communicated to provide a fair criminal investigation.

The use of GC-MS and LC-tandem MS (MS/MS) for detection are now very much common place within hair analysis, with laboratories moving towards dual MS systems such as LC-MS/MS due to greater sensitivity. The instrumentation used can still be quite varied (i.e., different ionizations or mass analysers), with successful applications that have included: LC-electrospray ionization-MS/MS (Chèze et al., 2004; Maublanc et al., 2014; Míguez-Framil et al., 2011; Miller et al., 2006), LC-atmospheric pressure chemical ionization-MS/MS (Kłys et al., 2007), two-dimensional GC-TOF-MS/MS (Guthery et al., 2010), UPLC-MS/MS (Di Corcia et al., 2012; Johansen & Dahl-Sørensen, 2011; Kim et al., 2015; Ramírez Fernández et al., 2015) (See Table 3).

Imaging techniques for the detection of the spatial distribution of drugs in hair has more recently been investigated (Kamata et al., 2015; Shen et al., 2014). Of particular note is the use of MALDI-TOF, MALDI-Fourier transform ion cyclotron resonance and MALDI-MS (Cuypers et al., 2016; Miki et al., 2011). Such techniques show promise for high sensitivity detection of low concentrations of drugs in hair segments, while removing the longer sample preparation procedures required with traditional techniques such as GC-MS and LC-MS/MS (Porta, Grivet, Kraemer, Varesio, & Hopfgartner, 2011). Imaging techniques would provide a more rapid initial identification of whether any drugs were present before further analysis, and as displayed by Cuypers et al. (2016), imaging has provided enhanced knowledge of an incorporation route of cocaine contaminants from the hair surface into the hair matrix during the wash procedure.

4.5 | Data interpretation

Of particular importance when interpreting hair analysis data, is the acknowledgement that drug chemistry and metabolism can often be quite complex. Pavlic, Libiseller, Grubwieser, Schubert and Rabl (2006) observed that tetrazepam was metabolized to diazepam within the body, then diazepam further metabolized to nordiazepam, indicating that it is easy to misinterpret the results if knowledge of drug metabolism is lacking, while the reporting of the incorrect parent drug could lead to serious consequences for either the victim or the accused within a legal setting. Therefore, it is important to consider the proportion of the metabolites detected, which is important for the analysis of new psychoactive substances (NPS) that have recently entered the illegal market. NPS are often derivatives, structurally similar to, or metabolites of existing drugs, and can display similar or overlapping metabolic pathways to their traditional counterparts, making their interpretation and identification extremely problematic (Partridge, Trobbiani, Stockham, Charlwood, & Kostakis, 2018).

The use of limit of detection cut-off values has been previously reported, and the SoHT provides a list of cut-off values for various drugs (Cooper et al., 2012). The detection of a drug above the cut-off values can be reported as a positive detection. However, these values are normally only applicable for chronic use of a drug, as a single drug consumption may fall below the cut-off value. The proposed list of recommended cut-off values cover some of the most commonly encountered drug groups, e.g., amphetamines (0.2 ng/mg), cannabinoids (0.1 ng/mg), cocaine (0.5 ng/mg), opiates (0.2 ng/mg), methadone (0.2 ng/mg) and buprenorphine (0.01 ng/mg) (Cooper et al., 2012). These recommended cut-off values vary due to the chemical differences between drugs. Although the purpose of these values is to reduce false positive interpretation of a detected drug or metabolite, caution should be exercised with their use for two important reasons, i.e., (a) SoHT (2004) noted that cut-off values may be misleading, as external drug contamination can be at any concentration and therefore challenging to interpret (e.g., surface and passive contamination from cannabis and crack cocaine smoke), and (b) detected concentrations may fall well below the accepted cut-off limits when a single exposure has been performed, for example within DFC. It should also be noted that cut-off values are drug specific, with many drugs having no published or accepted values.

Importantly all procedures used should be validated for bias, precision, accuracy and robustness. However, it is the responsibility of the laboratories to prove the reliability of the validation process for each use Scientific Working Group for Forensic Toxicology, 2013). The SoHT also encourages the joining and use of a laboratory proficiency testing scheme so as to keep best practice procedures up to date (Cooper et al., 2012). Overall, the analyst should be competent and trained with the use of each method, with an understanding of the interpretation processes.

5 | CONCLUSION

Hair analysis is a powerful tool within forensic toxicology. However, its usefulness is limited by the extent to which knowledge is available, and whether the samples and data are analysed and interpreted correctly. This cannot be stressed more than when it is applied to DFCs

in a court of law. In light of these points, we have discussed and proposed that it is possible to categorize key variables that affect hair analysis into three groups, with each group having a subsequent effect upon the next. While stages 1 and 2 contain unavoidable variables that are outside of the control of the analyst, their effects upon the controllable processes highlighted in stage 3 should not be ignored. As these variables can affect the outcome of hair analysis results, it is important to consider them to provide fair and just conclusions, while ruling out any bias and influencing factors. The techniques used for hair analysis vary between laboratories, and the standardization of the controllable stage 3 processes would be a step towards making data comparable. Taking into consideration the key variables that we have discussed in the three-stage approach should facilitate forensic toxicologists, hair analysis experts, judiciaries and service users in the interpretation of hair analysis results.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

ORCID

Christopher Davies https://orcid.org/0000-0003-3184-0208
Lata Gautam https://orcid.org/0000-0002-0409-4724

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How to cite this article: Davies C, Gautam L, Grela A, Morrissey J. Variability associated with interpreting drugs within forensic hair analysis: A three-stage interpretation. *J Appl Toxicol*. 2020;40:868–888. https://doi.org/10.1002/jat.3959