**Long title:**

**Variability Associated with Interpreting Drugs within Forensic Hair Analysis: A Three Stage Interpretation**

**Short title:**

**Three Stage Consideration for Hair Analysis Results Interpretation**

**Authors Names:**

\*Christopher Davies1, Lata Gautam1, Agatha Grela2, Joanne Morrissey1

1Forensic and Investigative Studies, School of Life Science, Faculty of Science and Engineering, Anglia Ruskin University, Cambridge, CB1 1PT

2School of Human and Social Sciences, University of West London, Brentford, London, TW8 9GA

**Corresponding Author**: christopher.davies3@pgr.anglia.ac.uk

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**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 inter-personal variability. At present there is little standardisation 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 categorised these variables and proposed a three-staged analytical approach to facilitate forensic toxicologists, hair analysis experts, judiciaries and service users in the analytical and interpretation process.

**Short Abstract**

The analysis of drugs in hair has the potential to be used as a powerful source of forensic evidence. The biological processes involved however, are still not fully understood. This paper discusses the factors that can influence the outcome of hair analysis results and suggests a three-stage interpretation of the many variables that an analyst should consider when presenting analytical data.

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 and drug facilitated sexual assaults (Rossi, et al., 2009; Kim et al., 2013; Chatterton & Kintz, 2014; Kim et al., 2015).

Many drugs associated with drug facilitated crimes (DFC) and drug facilitated sexual assaults (DFSA) exhibit short metabolic half-lives, being rapidly removed from the body, and becoming quickly undetectable (Kintz, et al., 2005a; Carter, 2010; Gautam et al., 2014). Blood and urine sampling therefore, 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 et al., 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. There are, nevertheless, 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, standardisation, or agreement on the analytical processes, making the comparison of data between laboratories challenging. There are many variables that may interfere with the outcome of results that can be easily overlooked. Data analysis should recognise 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, et al. 2011), drug incorporation routes (Harkey, et al., 1993; Poletini et al., 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. It is therefore 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 categorised 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 one influences stage two, and stage two influences stage three, the only variables that are within the control of the analyst are within the third i.e. final stage. These stages should therefore be considered when interpreting drug hair analysis results, and the significance of variables expressed within the findings.

1. **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 inter-person 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 et al. 2004a). Bromazepam was detected at 0.8 and 4.7 pg/mg respectively in the proximal 0-2 cm segments. In a similar study, Cheze et al. (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 same in these studies.

In two other notable studies, volunteers were administered 10 mg doses of zolpidem (Villain et al., 2004b; Cui et al., 2013). 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 et al., 2004b and 20 Chinese volunteers/Cui et al., 2013) and other factors such as inter-individual variations, such as metabolism, age, gender, incorporation rates, and hair colour. Shen et al. (2013) identified that the Chinese Han population were poor metabolisers 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 post administration 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). This however, still cannot account for the 100-fold increase of zolpidem concentration in hair observed between these studies.

Sato et al. (1993) however, was 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, Polletini et al (2012) administered controlled doses methamphetamine, identifying a within-subject correlation of dosage with detected concentrations, and that the detection also 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 et al. (1997a) was that conjugation of drug functional groups by either hydrolysis, reduction, and oxidation may occur to a minor extent within the hair follicle, leading to greater drug polarity. The authors demonstrated that there is a presence of many metabolic enzymes that are able to catalyse phase I and phase II reactions within the hair follicle itself, however, these are likely to be present in very small concentrations. Such compounds detected include; NADPH-P-450-reductase, P-450-aromatase, P-450-O-deethylase, along with alcohol dehydrogenase, carboxylases, esterase D, glutathion-reductase, glycosyl-transferase, glutathione-S-epoxide-transferase, and sulfotransferase (Pötsch et al., 1997). This area would benefit from more investigation as the presence of several cytochrome P-450 isozymes are important for the metabolism and biotransformation of many drugs (Ogu & Maxa, 2000).

Drug metabolism on a whole, varies markedly between individuals, indicating that accurate dose–hair concentration determinations are unlikely. Such inter-variation 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 metabolise 25% of clinical drugs. It is also found in smaller quantities within Caucasian individuals than in other 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 & Scott, (2006) suggest that the physiochemical properties of a drug are more important than its plasma concentration.

1. **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 that is still not understood, with knowledge advancing very little over the last 40 years, it must therefore 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 et al. (1993) hair contains 65-95% protein, 15-35% water, and 1-9% lipids. This 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, keratinisation, and melanin production. The cuticle, the outermost layer consists of overlapping dead keratinised cells approximately 0.5 µm thick. The cuticle, constructed of two layers, (i) the epicuticle outer layer - a lipoprotein membrane (Swift & Smith, 2001; Lyman & Schofield, 2008), and (ii) a high cysteine containing inner cuticle (Yang et al., 2014). The cortex, (the hair fibre core) resides beneath this (Yang et al., 2014) and sub-divided into three regions, (i) the orthocortex, (ii) paracortex, and (iii) mesocortex. Melanin is found within the cortex, and is responsible for hair pigmentation (Harkey et al., 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). 85% of hairs on the adult head are within the growth stage (anagen phase) at any one time, lasting between four to eight years with a rate of between 0.22 – 0.52 mm per day (Kintz, 2016), or 1.06 cm per month (Le Beau et al., 2011). The mean growth rate has become accepted as 1 cm per month (Pragst & Balikova, 2006; Kintz, 2013), however, Pragst & Balikova. (2006) state that a more accurate value would be 1.1 ±0.2cm/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 two 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 inter-person variation, knowledge of the rate of hair growth is important in hair analysis, most notably for segmental hair analysis (Section 4.3) where its growth rate allows for an approximate timeline of when the drug exposure took place. Harkey et al. (1993) described the now accepted average hair growth rate 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 et al., 2005a; Le Beau et al., 2011). Studies have investigated growth rates of human hair with various results. Table 2 displays fifteen studies between the years of 1951 to 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 summarised 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; Poletini et al., 2012), drug containing excretions from the sebaceous glands passing into hair during growth within the follicle (Harkey et al., 1993), the incorporation of the drug and metabolite into the hair follicle from sweat and sebum during growth (Harkey et al., 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 et al., 1996; Testorf et al., 2001). Larsson & 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 (Larson & 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 (Slawson, Wilkins & Rollins, 1998); Pragst, et al., 2006; Polettini et al., 2012). 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 (Potch et al., 1997a, Kronstrand et al., 2006; Pragst & Balikova, 2006; Yu et al., 2017). Sato et al. (1993) observed notable differences in the detected quantities of chlorpromazine in pigmented and non-pigmented hair using High Performance Liquid Chromatography (HPLC) after controlled doses were administered to volunteers. Detection in non-pigmented hair was found to be less than 10% of the observed amounts in black hair. The authors described detection in non-pigmented hair as much lower and even undetectable. In contrast to these findings, Kelley et al. (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 et al. (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 characterisation 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, the ratio of ionised to non-ionised drugs, pH gradient, blood flow, and the concentration gradient (Pötsch et al., 1997a). 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, and N-alkyl chains and a 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) the hair bulb, and (ii) the keratinised upper dermis. Further to this study, 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 keratinised upper dermis, while non-pigmented 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 (Nakahara et al., 1995; Kintz. 2012a; 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.

**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 minimise 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 et al., 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 et al. 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 (Section 3.4) (Salomone et al., 2012; Kintz, 2013; Wang et al., 2016).

The photosensitivity and effect of UV light on some drugs in hair should be also interpreted with care (Sato et al., 1993). The authors suggested that since 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, EDDP, 6-MAM, morphine, cocaine, and benzoylecgonine within positive hair samples when exposed to UVB radiation at 300 J/cm2. The authors noted that the drugs degraded by an average of 55%, 17%, 21%, 17%, 20% and 11% respectively. This importantly 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 et al., 1997). Volunteers with bleached and dyed hair, that 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-Mass Spectrometry. 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. It does however have limitations, especially with regards to 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 around 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). It has therefore 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 cm, 5-6 cm, and 6-8 cm). These findings suggest consumption on more than one occasion, however the authors also proposed other considerations; (i) thermal or cosmetic damage of the hair, (ii) swelling of the hair and an increase in water absorption capacity due to damage, aiding the migration of drug along the hair shaft, and (iii) incorporation of the drug during the growth cycles of hair.

1. **Stage 3 Variables: The Analyst and the Method**

**4.1. Variation Associated with Hair Sample Collection and Processing**

Stage three 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 standardised method and different techniques have been applied. The SoHT (Society of Hair Testing) (Cooper et al., 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 four to six 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 prior to cutting should be noted and written on the collection envelope.

Le Beau et al. (2011) studied the accuracy of hair collection and cutting techniques of fourteen 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. The authors therefore recommend a period of eight weeks after ingesting a possible drug before collecting hair specimens, particularly when segmental analysis is required, stating that inconsistencies 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**

The consideration of what the best washing and decontaminating method is, has been described by Tsanaclis et al. (2018) as an issue that arouses controversy in hair analysis. The choice, however, is a crucial step for eliminating impurities and analytical interferences. The SoHT guideline outlines several purposes of hair washing that include; (i) the removal of hair care products, (ii) sweat, sebum, and skin cells, (iii) head lice, and (iv) other body fluids. Any decontaminating procedures must remove any external contamination without extracting the internal drugs and analytes in question. Tsanaclis & Wicks, (2007) recommended that a hair washing procedure should be both practical and should also be able 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; protic and non-protic. 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 et al., (2007) they had included a one-minute methanol wash to limit this extraction process. Non-protic solvents are described as more reliable since they do not swell the hair. However, the Society of Hair Testing 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), with the processes themselves ranging from applying single wash solvent (Crimele et al., 1997; Aleksa, et al., 2012;), to multiple wash solutions (Miller et al., 2006; Kłys et al., 2007; Harun et al., 2010), 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 et al., 2000), acetone and hexane (Licata et al., 2016), sodium dodecyl sulphate (Nakahara & Hanajiri, 2000; Favretto et al., 2011) and isopropanol (Montesano et al., 2014).

Dichloromethane washes with or without ultrasonification appear to be the most common wash procedures used (Jurado et al., 1997; Anderson et al., 2008; Fisichella et al., 2014; Maublanc et al., 2014). Duvivier et al. (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 (i) methanol followed by sodium dodecyl sulphate (SDS) wash, (ii) methanol, SDS followed by methanol wash, (iii) 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 et al. (2001) had evaluated four different hair decontamination methods for the detection of cocaine and its metabolites in rat hair. These methods were: (i) methanol, (ii) phosphate buffer at pH 6, (iii) phosphate buffer at pH 8, and (iv) isopropanol followed by phosphate buffer at pH 8, and (v) no wash at all. The study showed that each wash method varied in efficiency, and had also 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. This study importantly demonstrated 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 Mass Spectrometry (MetA-SIMS) techniques were utilised by Cuypers et al. (2016) to compare the spatial distribution of hair externally spiked with cocaine with hairobtained from cocaine users. The authors identified two important factors with regards to the reliability of the wash solvents dichloromethane, and acetone; (i) These solvents did not remove all of the cocaine surface contaminants, and (ii) MetA-SIM analysis revealed that the solvents may 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 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 et al. (2000). Highest concentration decrease from spiked hair was observed for amphetamine ( -41.0%) followed by THC (-36.3%). No change was reported for 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) that drugs and their metabolites are incorporated into the hair shaft while it grows, and (ii) that the 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 one-month window for drug exposure. As a guide, hair samples should be collected between 4-6 weeks after an alleged drug facilitated crime. 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 earlier, 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, the accuracy of the segmentation cutting process, axial migration, and the age of the individual to whom the hair belongs. Due to these variations, the estimated timeframes are limited to between one to two months, depending upon the size of the segment. The SoHT guidelines recommend segments of between 10 mm to 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 8 volunteers. Results indicated that one 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 six months of exposure, as they had also observed a decrease in detection when sampling after ten months.

Many case studies have had success with corroborating the timeframe of an incident (Villain, et al., 2004a; Johansen & Dahl-Sørensen, 2011. For example, Johansen & Dahl-Sørensen, (2011) described the successful use of segmental hair analysis within an alleged drug facilitated sexual assault 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 x 2 cm segments were analysed by ultra-performance liquid chromatography time-of-flight mass spectrometry (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 hrs for triazolam), which may have gone undetected from blood and urine sample.

Further to these observations, MALDI-IMS and time-course mass spectrometry imaging of two possible methoxyphenamine incorporation routes into hair (hair bulb and keratinised 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 (Shima et al., 2016; Shima et al., 2017; Kuwayama et al., 2018). Notably, Kuwayama et al. (2018) developed a micro-segmental 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, but does however 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 with regards to 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 inter-individual 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), solid phase extraction (SPE) (Anderson, et al., 2008), liquid-liquid extraction (LLE), methanolic, and acidic hydrolysis (Cirimele et al., 1996b), and micro-pulverised 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 standardising 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., 1996b).

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, so as to provide a fair criminal investigation.

The use of GC-MS and LC-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 ionisations or mass analysers), with successful applications that have included; LC-ESI-MS/MS (Cheze, 2004; Miller et al., 2006; Míguez-Framil et al., 2011; Maublanc et al., 2014), LC-APCI-MS/MS (Kłys et al., 2007), GCxGC-TOF-MS/MS (Guthery et al., 2010), UPLC-MS/MS (Johansen & Dahl-Sørensen, 2011; Di Corcia et al., 2012; Ramírez Fernández et al., 2015; Kim et al., 2015) (See Table 3).

Imaging techniques for detection of the spatial distribution of drugs in hair has more recently been investigated (Shen et al., 2014; Kamata et., al 2015). Of particular note is the use of MALDI-TOF, Matrix-Assisted Laser Desorption Ionization-Fourier Transform Ion Cyclotron Resonance (MALDI-FTICR), and MALDI-MS (Miki et al., 2011, Cuypers et al., 2016). 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 et al., 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 et al. (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. It is therefore 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’s 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 et al., 2018).

The use of limit of detection cut off values have 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, since 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; 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) The Society of Hair Testing, (2004) noted that cut off values may be misleading since external drug contamination can be at any concentration, and therefore challenging to interpret, for example surface and passive contamination from cannabis and crack cocaine smoke, and (ii) the detected concentrations may fall well below the accepted cut-off limits when a single exposure has been performed, for example within drug facilitated crime. 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 laboratories responsibility to prove the reliability of the validation process for each use (Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology, 2013). The Society of Hair Testing 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.

1. **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 or not the samples and data are analysed and interpreted correctly. This cannot be stressed more than when it is applied to drug facilitated crimes in a court of law. In light of these points, we have discussed and proposed that it is possible to categorise key variables that affect hair analysis into three groups, with each group having a subsequent effect upon the next. While stages one and two contain unavoidable variables that are outside of the control of the analyst, their effects upon the controllable processes highlighted in stage three should not be ignored. As these variables can affect the outcome of hair analysis results, it is important to consider them so as 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 standardisation of the controllable stage three processes would be a step towards making data comparable. Taking into consideration the key variables that we have discussed in the three-staged approach should facilitate forensic toxicologists, hair analysis experts, judiciaries and service users in the interpretation of hair analysis results.

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**Table 1.** Stages Leading to Variability in Hair Analysis

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| **STAGE 1: Drug - Pharmacokinetic Interactions** |
|   | **UncontrollableVariables** |
| • The drug dosage administered |
| • How the drug was administered |
| • How often the drug was administered. |
| • Pharmacokinetic parameters of the drug |
| • Variation in metabolism between individuals |
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| **STAGE 2: Drug and Metabolite - Hair Interactions** |
|   | **UncontrollableVariables** |
| • 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 |
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| **STAGE 3: The Analyst and the Analytical Process** |
|   | **ControllableVariables** |
| • Sample collection and storage |
| • Decontamination and wash/solvent choice |
| • The analytical technique |
| • Analysis and data interpretation |
|   |

**Table 2**. Human Hair Growth Rates from Previous Studies

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| --- | --- | --- | --- |
| **Minimum** | **Maximum** | **Mean** | **Reference** |
| **(cm/month)** | **(cm/month)** | **(cm/month)** |  |
| 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 et al., 2015 |
| 0.70 | 1.40 | **1.05** | Kintz et al., 2013 |
| Not Known | Not Known | **1.09 ±0.20** | Scott et al., 2009 |
| 0.73 | 1.48 | **1.11** | Tajima et al., 2007 |
| 0.84 | 1.41 | **1.13** | Pragst et al., 2004 |
| 0.95 | 1.12 | **1.04** | Van Neste et al., 2004 |
| 0.65 | 2.20 | **1.43** | Pötsch et al., 1996 |
| 0.60 | 3.36 | **1.98** | Harkey et al., 1993 |
| 0.90 | 1.20 | **1.05** | Bost et al., 1993 |
| 0.84 | 1.37 | **1.11** | Miyazawa & Uematsu, 1992 |
| 0.76 | 0.96 | **0.86** | Pecoraro et al., 1964 |
| Not Known | Not Known | **0.94** | Barman et al., 1964 |
| 0.84 | 1.15 | **1.00** | Myers & Hamilton, 1951 |

**Table 3** Summary of methods used in drugs detection from hair

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Washing / Decontamination** | **Buffer Solutions** | **Extraction** | **Analysis** | **Analyte(s)** | **Reference** |
| Detergent x3, water x3 | Phosphate buffer pH 7.4 | Pulverised, methanol (heated for 2 hours), centrifugation, evaporation | RIA7 | Heroin and MOR25 metabolites | Baumgartner et al., 1979 |
| Not described | Tris buffer pH 7.1 | Toxi-tube ADigested with pronase and dithiothreitol | GC-MS8 | COC24, BZE26, EME27, MOR25, COD28, 6-MAM29, | Barrera et al., 1995 |
| Dichloromethane x2 (2 min) | Sorensen phosphate buffer pH 7.6 (40°C for 2 hours) | Diethylether/chloroform (80/20, v/v), agitated (20 min), centrifugation (15 min) | GC-MS-NCI9 | Flunitrazepam | Cirimele et al., 1996b |
| Dichloromethane x2 (2 min) | Sorensen phosphate buffer pH 7.6 (40°C for 2 hours) | Diethylether/chloroform (80/20, v/v), agitated, centrifugation (15 min) | GC-MS-NCI9 | Flunitrazepam, 7-aminoflunitrazepam | Cirimele et al., 1997 |
| Ethanol/methanol (9/1, v/v) (3 min) | With and without Acetate buffer pH 4 | Multiple:(1) Methanol, (2) Aylsulfatase/β-glucuronidase SPE1(3) Aqueous urea solution SPE1, (4) Acetone, (5) 2-propanol | GC-MS8 | COC24, BZE26, MOR25, MAM31 | Eser et al., 1997 |
| Dichloromethane x2 (15 min) | None | HCl21, organic solvent | GC-MS8 | MOR25, BZE26, COD28, 6-MAM29, THC32, THC-COOH33, nicotine | Jurado et al., 1997 |
| Sodium dodecyl sulphate x3, water x3 (Rinsed) | None | Methanol/HCl21 (20/1, v/v, sonification for 1 hour), trifluoroacetic anhydride/ethyl acetate (1/1, v/v, 60°C for 20 min) | GC-MS8 | 7x MAMPs35 | Nakahara & Hanajiri, 2000 |
| Methanol (1 hour sonification) | None | Methanol (sonification 1 hour), centrifugation (5 min), HCl21 (vortexed 55°C overnight), acetic acid and water, SPE1  | GC-MS8 | Clonazepam, 7-aminoclonazepam | Negrusz et al., 2000 |
| Methanol (sonification 1 hour + centrifugation for 5 min)) | None | Methanol, water, acetic acid, HCl21, (Dichloromethane, isopropanol, ammonium hydroxide 78/20/2, v/v), SPE1 | GC-MS8 | 7-aminoclonazepam, clonazepam | Negrusz et al., 2002 |
| Sodium dodecyl sulphate and water x3 | Multiple compared | Multiple extraction methods compared; (i) Proteinase K, (ii) Basic methanolic, (iii) Acidic methanolic, (iv) Soerensens buffer (v) NaOH22, (vi) β-Glucuronidase, (vii) Protease | HPLC10 and GC-MS8 | BZPs36 | Scott & Nakahara, 2003 |
| Dichloromethane x2 (5 min) | Sorensen buffer pH 7.6 (56˚C for 14 hours) | Dichloromethane, LLE2 (10 min), centrifugation | LC-MS/MS11 | Bromazepam, clonazepam, metabolites | Cheze et al., 2004 |
| Dichloromethane | Phosphate buffer pH 8.4 (overnight) | Dichloromethane/diethyl ether (80/20, v/v), centrifugation (15 min) | LC-MS/MS11 | Lorazepam | Kintz, et al., 2004 |
| Dichloromethane | Phosphate buffer pH 8.4 (overnight) | Dichloromethane/diethyl ether (90/10, v/v), agitation and centrifugation (15 min) | LC-MS/MS11 | Tetrazepam | Concheiro et al., 2005 |
| Dichloromethane x2 (2 min) | Phosphate buffer pH 8.4 (overnight) | Dichloromethane/diethyl ether (80/20, v/v) | LC-MS/MS11 | Alprazolam | Kintz et al., 2005b |
| Dichloromethane x2 (2 min) | Phosphate buffer pH 8.4 (overnight) | Dichloromethane/diethyl ether (90/10, v/v), agitation (15 min), centrifugation (15 min) | LC-MS/MS11 | 16x BZPs36, hypnotics | Villain et al., 2005 |
| Aqueous sodium dodecyl sulfate x1 (10 min ultrasonification), deionised water x2 (10 min ultrasonification), dichloromethane x2 (10 min ultrasonification) | Phosphate buffer pH 6.0 | Methanol/NaOH22 (20/1, v/v, sonification for 1 hour, then left overnight), second wash performed with methanol | ELISA13 and LC-ESI-MS/MS12 | BZPs36 | Miller et al., 2006 |
| Not explained | Ammonium chloride pH 9.2 | Methanol (45°C shaking for 2 hours), 1-chlorobutane, formic acid, water/methanol (70/30, v/v) | LC-MS/MS11 | 26x BZPs36 | Laloup et al., 2007 |
| Water and acetone (x2) | None | Methanolic HCl21 (50°C ultrasonification for 1 hour) | GC-MS8 | Phenylalkylamine derivatives | Kim et al., 2007 |
| n-hexane (vortexed 30 sec), acetone (vortexed (30 sec) | None | Methanol (vortexed and ultrasonification at 50°C for 1 hour, left overnight) | LC-APCI-MS/MS14 | OPIs37, AMPs38, COCs24 | Kłys et al., 2007 |
| Shampoo, water, dichloromethane, isopropanol, acetone | Phosphate buffer pH 7.0 and potassium hydroxide pH7.0 | HCl21 (50°C for 16 hours), SPE1 (methanol and water and phosphate buffer pH 7.0),  | GC-MS8 | OPIs37, AMPs38, COC24, diazepam, metabolites | Cordero & Paterson, 2007 |
| Aqueous sodium dodecyl sulfate x1 (10 min ultrasonification), deionised water x2 (10 min ultrasonification), dichloromethane x2 (10 min ultrasonification).  | Phosphate buffer pH 6.0 for SPE1, toluene for MISPE4 | Methanol/ammonium hydroxide (20/1, v/v), ultrasonification (1 hour, left overnight), second wash of methanol/ammonium hydroxide (20/1, v/v), SPE1 and MISPE3 compared | LC-MS/MS11 | BZPs36 | Anderson et al., 2008 |
| Soap x3 (10 min), water x3 (rinsed) | None | Microwave Assisted Extraction (Methanol, 60°C for 9 min) | HPLC10 | OPIs37, COC24 and metabolites | Fernández et al., 2009 |
| Dichloromethane x3 | Formic acid pH 4.0 | Methanol, ultrasonification (40°C for 4 hours) | LC-MS/MS11 | MOR25, COD28, 6-AM30, BZE26, COC24 | Huang et al., 2009 |
| Water (shaking 4 min), acetone (shaking 4 min), petroleum ether (shaking 4 min) | None | Extracted twice: Alkaline hydrolysis then LLE2, and methanolic extraction | GC-MS8 | Δ9-THCA34, THC32 | Auwärter et al., 2010 |
| Shampoo, sodium dodecyl sulphate, water, dichloromethane | Phosphate buffer pH5.0 (45°C for 18 hours) | Methanol/NaOH22, MISPE3 (acetonitrile and phosphate buffer pH 5.0, vortexed, eluted in 30% acetic acid in acetonitrile) | LC-MS/MS11 | KET39, norKET40 | Harun et al., 2010 |
| Methanol x3 | Phosphate buffer pH 7.0 | HCl21 (37°C for 16 hours), vortexed and centrifugation (5 min), SPE1 | GCxGC-TOF-MS15 | Multiple | Guthery et al., 2010 |
| Methanol x4  | Acetonitrile pH 5.3 | Methanol/acetonitrile/ammonium formate (25:25:50, v/v/v), centriugation (10 min), incubation (37°C for 18 hours), centrifugation (10 mins)  | UPLC-TOF-MS16 | 52x pharmaceutical, and drugs of abuse | Nielsen et al., 2010 |
| Sodium dodecyl sulphate x1 (3 min), water x2 (3 min), acetone x1 (3 min) | Phosphate buffer pH 4.0 | Methanol/TFA (90/10, v/v), ultrasonification (1 hour), 45°C overnight, Dichloromethane/diethyl ether LLE2 | HPLC-HRMS17 | AMPs38, COC24, OPIs37, BZPs36, antidepressants, hallucinogens | Favretto et al., 2011 |
| Methanol, water/methanol x2 | None | Methanol (38°C for 16 hours) | LC-MS/MS11 | 27x BZPs36 and zolpidem | Kim et al., 2011 |
| Water x1, methanol x1 | Phosphate buffer pH 6.0 | Methanol (38°C for 16 hours), SPE1  | GC-MS8 | Hypnotics | Lee et al., 2011 |
| Dichloromethane x3 (2 min) | Borate buffer pH 9.0 | Acetonitrile (50°C for 12 hours), centrifugation (10 min at 4°C), then two step extraction; LLE2 and SPE1 | LC-MS/MS11 | Multiple, 35 drugs and metabolites | Lendoiro et al., 2011 |
| Soap solution, water (Stirred for 30 min) | Sodium borate 2M/boric acid 2M (50/50, v/v) | MSPD4, SPE1 | ESI-MS/MS18 | OPIs37, COC24 | Míguez-Framil et al., 2011 |
| 0.5% Sodium dodecyl sulphate x1Distilled Water x3 (ultrasonification 1 min)Methanol x3 (ultrasonification 1 min) | Not Required | Not Required | MALDI-TOF-IMS, and MALDI-FTICR-IMS | MAMP35 | Miki et al., 2011 |
| Dichloromethane x2 (Vortexed 3 min) | Formic acid buffer | Methanol (55°C for 15 hours) | LC-MS/MS11 | Zolpidem and multiple | Salomone et al., 2012 |
| Dichloromethane x2 (rinsed) | Phosphate buffer pH 8.4 (ultrasonification 1 hour) | LLE2 (dichloromethane) | LC-MS/MS11 | 18x BZPs36 | Xiang et al., 2011 |
| Dichloromethane x2 (1 min) | Phosphate buffered saline pH 7.4 | Methanol (agitation, 56°C for 18 hours), HS-SPME5 | GC-MS8 | Multiple, 17 drugs and metabolites | Aleksa et al., 2012 |
| Dichloromethane x2 (vortexed 3 min) | None | Methanol (55°C for 15 hours) | UHPLC-MS/MS19 | Multiple | Di Corcia et al., 2012 |
| 2-propanol (ultrasonification, 5 min), aqueous soln x3 (ultrasonification 5 min) (washed after segmentation) | None | Centrifugation (20°C for 10 min), incubated (37°C for 18 hours), centrifugation (10 min) | UPLC-MS/MS20 | Triazolam and metabolites | Johansen & Dahl-Sørensen, 2012 |
| Dichloromethane (vortexed 30 sec), methanol (vortexed 30 sec) | None | HCl21 (brief vortex and centrifugation), incubated (45°C overnight), centrifugation (1 min), water (centrifugation 10 min) | LC-MS/MS11 | COC24 and metabolites | Alves et al., 2013 |
| Methanol, water/methanol x2 | None | Methanol (38˚C for 16 hours), SPE1 | LC-MS/MS11 | BZP36 and zolpidem | Kim et al., 2013 |
| Dichloromethane x2 (2min) | Ammonium chloride buffer pH 9.5 (40°C overnight) | Incubated (45°C overnight w/buffer), LLE2 (Dichloromethane/isopropanol/n-heptane (25/10/65, v/v)) | LC-MS/MS11 | Amitriiptyline, temazepam, tramadol, dihydrocodeine | Chatterton et al., 2014 |
| Dichloromethane, methanol | None | Methanol, ultrasonification (1 hour) | LC-MS/MS11 | 87x psychoactive drugs | Fisichella et al., 2014 |
| Water x 2 (2min), dichloromethane x2 (1min) | Phosphate buffer pH 5 (shaking for 16 hours) | NaOH22, 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/MS12 | BZPs36, hypnotics, psychotropics | Maublanc et al., 2014 |
| Isopropanol x1, water x2 (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 hours) | UPLC-MS/MS20 | 96x drugs (multiple classes) | Montesano et al., 2014 |
| Methanol x2, water x2, methanol x2 | None | Methanol (Stirring 38°C for 16 hours) | LC-MS/MS11 | NNEI41, MAM-220131 | Saito et al., 2014 |
| Wiped with tissue paper moistened with distilled water | AcetatepH 5.0 | Pulverised with acetate buffer (5 min), ultrasonification (30 min), LLE2 with Chloroform-Isopropanol (3/1, v/v), organic layer seprated. | LC-MS/MS | MOP47 | Kamata et al., 2015 |
| Wiped with tissue paper moistened with distilled water | Not Required | Not Required | MALDI-TOF-IMS | MOP47 | Kamata et al., 2015 |
| Methanol x2, water x2, methanol x2 | None | Methanolic extraction (stirring 38°C for 16 hours) | UHPLC-MS/MS19 | Multiple | Kim et al., 2015 |
| Water (2 min), acetone (2 min) | Ammonium acetate pH 4.0 | Methanol (sonification 50-60°C for 4 hours), formic acid | LC-MS/MS11 | MDMA42, MDA43 | Madry et al., 2015 |
| Dichloromethane, water | Phosphate buffer | Methanol (sonification 45°C for 2 hours), LLE2 (1-chlorobutane) | UHPLC-MS/MS19 | BZPs36 and Z-Drugs | Ramírez Fernández et al., 2015 |
| Acetone x2, Hexane x2 | None | Methanol (45°C overnight), roQdSPE QuECheERS6 sorbent kit | LC-MS/MS11 | 50x psychoactive drugs and metabolites | Licata et al., 2016 |
| Water x1 (1 min), acetone x1 (1 min) | Ammonium formiate pH 5.3 | Methanol/acetonitrile/ammonium formiate (25/35/50, v/v), centrifugation | LC-MS/MS11 | EME27, MOR25, COD28, AMP38, 6-MAM29, MAMP35, MDMA42, BZE26, MDEA44, COC24, EDDP45, MTD46 | Cappelle et al., 2017 |
| None Described | None described | Ammonium acetate w/0.5% formic acid/MeCN23 (3/1, v/v), sonification (10 min), room temperature (24 hours) | LC-MS/MS11 | Dyphenhydramine | Kuwayama et al., 2018 |

**Extraction**: 1Solid-phase extraction, 2Liquid-liquid extraction, 3Molecularly imprinted solid-phase extraction, 4Matrix solid-phase dispersion 5Headspace solid-phase microextraction, 6Quick Easy Cheap Effective Rugged and Safe.

**Instrumentation**: 7Radioimmunoassay, 8Gas chromatography-mass spectrometry, 9Gas chromatography-mass spectrometry-negative chemical ionisation, 10High pressure liquid chromatography, 11Liquid chromatography-tandem mass spectrometry, 12liquid chromatography-electrospray ionization-tandem mass spectrometry, 13enzyme-linked immunosorbent assay, 14liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry, 15two-dimensional gas chromatography-time of flight-mass spectrometry, 16Ultra performance liquid chromatography-time of flight-mass spectrometry, 17High performance liquid chromatography-high resolution mass spectrometry, 18Electrospray ionisation-tandem mass spectrometry, 19Ultra high performance liquid chromatography-tandem mass spectrometry, 20Ultra performance liquid chromatography-tandem mass spectrometry.

**Compounds**: 21Hydrochloric acid, 22Sodium hydroxide, 23Acetonitrile, 24Cocaine, 25Morphine, 26Benzoylecgonine, 27Ecgonine methyl ester, 28Codeine, 296-Monoacetylmorphine, 306-acetylmorphine, 31Monoacetylmorphine, 32 Δ9-tetrahydrocannabinol, 3311-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid, 34Δ9-tetrahydrocannabinolic acid A, 35methamphetamine, 36Benzodiazepines, 37Opiates, 38Aphetamine, 39Ketamine, 40Nor-Ketamine, 41N-1-naphthalenyl-1-pentyl-1H-indole-3-carboxamide, 42Methylenedioxymethamphetamine, 43methylenedioxyamphetamine, 44methylenedioxyethylamphetamine, 45ethylenedimethyldiphenylpyrrolidine, 46Methadone, 47Methoxyphenamine.