Assessment of incorporation of drugs into hair, extraction and analysis techniques, and potential interferences with hair analysis for drug detection

by

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Declaration

'I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.'

Caitlyn A. Rogers

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Summary

The analysis of drugs from hair samples can provide forensic toxicologists a great deal of information that cannot be gained through the analysis of blood and urine. Hair analysis is usually performed as a complementary technique to blood and urine analysis, but has the added advantage of having a greater detection window (months to years, compared to hours to days). This longer detection window allows for determination of chronic drug use, chronic poisoning, the monitoring of drug abstinence and obtaining a drug history. In forensic toxicology, hair analysis can be used in cases involving drug facilitated crimes (DFC's) including drug-facilitated sexual assault, child protection and suspicious death. Despite this, there are no recognised standard methods for hair analysis. Methods of extracting the drugs from hair are of particular concern, and can vary significantly from lab to lab. Extraction generally involves an incubation step which can last for up to 18 hours. This lengthy extraction process can be a hindrance to case flow. Additional problems with the hair analysis can arise if the hair has been cosmetically treated after a drug has been consumed. Furthermore, authentic drugged hair samples (i.e. hair from a donor that has been administered drugs) should be used for method development and validation. However, it is difficult to acquire authentic hair samples with enough variety of drugs at high enough concentrations to use for this purpose. Artificially incorporating drugs into hair has been proposed for method development and R&D purposes involving hair analysis for drug detection. However, very little is understood about the underlying factors that influence the incorporation. For example, how does solvent affect the degree of drug incorporation into the hair shaft.

Research presented in this thesis examines alternative methods of extraction as a potential substitute for an 18-hour incubation process used for the analysis of hair, along with an investigation into the effects of cosmetic hair treatments on drug recovery. Finally, investigations into the preparation of artificially spiked hair samples for use in method development are presented.

Microwave-assisted extraction (MAE) into methanol resulted in extraction time being reduced, without the requirement for any change to the sample preparation process. The

extraction time and temperature were optimised with the optimum conditions being microwaving for 2 hours at 70 °C for a 20 mg hair sample in 2 mL solvent. Other variables were unable to be studied, as the research was limited by the capacity of the microwave which could only irradiate one sample at a time.

The use of an ionic liquid composite (ILC) to disassemble the structure of hair prior to extraction was also assessed. There is has been no prior work published on the use of ionic liquids, or ionic liquid composites, for the digestion and extraction of drugs from hair. The ILC was shown to disassemble the hair structure, however the high temperatures required and the viscosity of the ILC are unsuitable for extraction and detection of drugs in forensic analysis. There is however, potential for this ILC to be used in the detection of heavy metals and other thermally stable compounds in hair.

The effects of three cosmetic hair treatment protocols on drug recovery and hair structure were investigated. These were i) Olaplex® treatment, ii) bleach treatment, and iii) a combined Olaplex® – bleach treatment program. Olaplex® is a recently released cosmetic hair treatment reported to repair hair by rebuilding disulphide bonds. Marketing suggests that it will reduce the damage caused by additional treatments such as bleaching. While there are many cosmetic treatments that have previously been assessed on their potential to interfere with drugs in the hair, Olaplex® has not yet been investigated. There also no reports on how the treatment affects the hair structurally, at a microscopic level. Results presented in this thesis show that the Olaplex® treatment did reduce the damage done to the hair, when compared to bleaching alone. However, this treatment did not reduce the amount of drug lost during bleaching treatments. It was also found through the use of FT-IR that hair that had been bleached could be differentiated from naturally blonde hair. However, this method could not detect Olaplex® on Olaplex® treated hair.

Finally, an investigation into how temperature, solvent ratios (of methanol and DMSO), and prior cosmetic treatment affect the artificial incorporation of drugs into hair. While there have been a small number of reports on artificial incorporation of drugs into hair, very little detail is included in regard to processes, and none have investigated varying effects to incubation time and temperature. This study demonstrated that artificial incorporation is possible, and the level of incorporation can be altered through variation

of temperature and solvent ratios. Results show that higher temperatures result in better incorporation for most drugs studied. The methanol/DMSO ratio was also shown to influence the level of incorporation, with increased incorporation observed when the methanol/DMSO ratio was higher. Preliminary work into the use of TOF-SIMS for cross sectional analysis of the hair was unsuccessful. Thus, it was not possible in this study to determine how accurately the artificially incorporated hairs represented authentic hairs, and additional work is needed. Regardless, this work present new insights on how effectively drugs can be incorporated into hair.

Original contributions of knowledge

While Microwave-assisted extraction (MAE) is an emerging technique and has been previously used forensic and clinical toxicology, there is limited literature specifically relating to hair analysis. This research thoroughly investigates MAE in hair analysis. Additionally, extraction of stimulants from any biological matrix, let alone hair, had been investigated. This research investigated the extraction of four key stimulants (amphetamine, methamphetamine, ecstasy and cocaine), along with a number of benzodiazepine and opioids. Effective extraction of stimulants is vital in hair analysis as they account for some on the most heavily abused drugs. Methamphetamine is a particular concern here in South Australia.

Ionic liquids, and ionic liquid composites, have never been examined with regards to their applications to forensic toxicology. While this section of the project was not as successful as hoped, and in unlikely to become a viable method in toxicology, it is entirely new work. This investigation also leads to the possibility of using ionic liquids and ionic liquid composites for extraction and analysis of heavy metals in hair. This has also not been investigated as yet.

It is well known that cosmetic treatments, such as bleaching, can cause quite a lot of damage to hair, and in turn cause issues with hair analysis. Cosmetic treatments such bleaching, dying, perming, chemical straightening, heat treatments (i.e. curling/straightening iron and blow drying) and ever use of styling products have all been investigated in depth with relation to effects on drug concentration in hair and potential interference with analytical techniques. However, Olaplex® is a relatively new product on the market that has not yet been investigated. This product is in high demand and is used in majority of salons around Australia, and internationally. There are now replicate products on the market mimicking the chemical makeup and function of Olaplex®. As such, it is vital to determine whether this this product has a detrimental effect on the drug concentration in hair or on the analysis of hair. The study presented in this thesis is the first to not only investigate to how the treatment effects the detectable drug concentration in hair, but also investigates the effect of the structural integrity of hair at

a microscopic level, and whether Olaplex® can be detected in hair case where treatment is suspected using ATR and FTIR.

Hair standards/reference materials are critical for method development and quality control in forensic laboratories. These standards however can be very expensive and hard to source and for this reason, some laboratories source and make their own reference material through artificial incorporation of drugs and metabolites into hair. However, has been no systematic study published on these processes. This thesis investigates the effects of temperature, solvent ratios and prior cosmetic treatments of the hair have on the quantity of drug that is incorporated into the hair shaft. This work present new insights on how effectively drugs can be incorporated into hair, and how procedures can be altered to suite the requirements of the reference material being produced. This work also shows a major flaw in the reference materials produced in this way, that has not previously been reported.

Contents

Tables

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Equations

Acronyms

µL	Microliter (1mL = 1000 µL)
AMP	Amphetamine
ANZFSS	Australian and New Zealand Forensic Science Society
ATR	Attenuated total reflection
BPC	Base Peak Chromatogram
COC	Cocaine
COD	Codeine
CNS	Central Nervous System
DE	Deep eutectic
DFC	Drug-facilitated crime
DFSA	Drug-facilitated sexual assault
DI	De-ionized
DIAZ	Diazepam
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDDP	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (metabolite of methadone)
EtG	Ethyl glucuronide
FAEE	fatty acid ethyl ester
FT-IR	Fourier-transform infrared spectroscopy
GHB	γ-Hydroxybutyric acid or γ-Hydroxybutyrate
HPLC	High-performance liquid chromatography (formerly referred to as high-pressure liquid chromatography)
ICP-MS	Inductively coupled plasma mass spectrometry
ILC	Ionic liquid composite
IR	Infrared
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography coupled with tandem mass spectrometry
LLE	liquid-liquid extraction

LOQ	Limit of Quantification
MAE	Microwave-assisted extraction
MALDI	Matrix Assisted Laser Desorption/Ionization
MAM	Monoacetylmorphine
MDMA	Methylenedioxymethamphetamine
MET	Methadone
METH	Methamphetamine
mL	Milliliter
MOR	Morphine
MW	Molecular weight
n-BuCl	Butylchloride
NOR	Nordiazepam
OXAZ	Oxazepam
OXY	Oxycodone
PDMS	Polydimethylsiloxane
pg/mg	Picagram per milligram (picagram of drug per milligram of hair)
QQQ	Triple Quadrupole mass spectrometer
QTOF	Quadrupole-Time of flight mass spectrometer
SD	Standard deviation
SEM	Scanning electron microscope
SoFT	Society of Forensic Toxicologists
SoHT	Society of Hair Testing
SPE	Solid-phase extraction
TCEP	Tris (2-carboxyethyl) phosphine
TIAFT	The International Association of Forensic Toxicologists
ToF-SIMS	Time-of-Flight Secondary Ion Mass Spectrometry
TOXSAG	Australian/New Zealand Specialist Advisory Group in Toxicology

Chemicals

CHEMICALS AND REAGENTS

Chemical name	**Chemical Formula**	**CAS No.**	**Source**
Acetonitrile	C_2H_3N	75-05-8	RCI Labscan
Ammonium hydroxide	NH_4OH	1336-21-6	Chem-Supply
Bleach Powder, containing:			
Potassium persulphate	$K_2S_2O_8$	7727-21-1	Hair Care
Ammonium persulphate	$(NH_4)_2S_2O_8$	7727-54-0	Australia
Sodium metasilicate	Na_2SiO_3	6834-92-0	
Sodium Stearate	$C_{18}H_{35}NaO_2$	822-16-2	
Magnesium carbonate	$MgCO_3$	546-93-0	
Choline chloride	$C_5H_{14}ClNO$	67-48-1	Sigma-Aldrich
DMSO	C_2H_6OS	67-68-5	Sigma-Aldrich
DTT	$C_4H_{10}O_2S_2$	27565-41-9	Sigma-Aldrich
Ethanol	C_2H_6O	64-17-5	Chem-Supply
Formic acid (0.1% in water)	CH_2O_2	64-18-6	Chem-Supply
Garnier® Fructis fortifying conditioner, containing:			
Water	H_2O	7732-18-5	Supermarket
Cetearyl alcohol	$CH_3(CH_2)_nCH_2OH$	67762-27-0	
Behentrimonium chloride	$C_{25}H_{54}ClN$	17301-53-0	
Starch acetate	$C_{29}H_{50}O_{21}$	9045-28-7	
Niacinamide	$C_6H_6N_2O$	98-92-0	
Benzyl alcohol	C_7H_8O	100-51-6	
Isopropyl alcohol	C_3H_8O	67-63-0	
Various plant and fruit extracts	N/A	N/A	
Garnier® Fructis fortifying shampoo, containing:			
Water	H_2O	7732-18-5	Supermarket
Sodium laureth sulphate	$CH_3(CH_2)_{11}(OCH_2CH_2)_nOSO_3Na$	9004-82-4	
Glycerine	$C_3H_8O_3$	56-81-5	
Glycol distearate	$C_{38}H_{74}O_4$	627-83-8	
Sodium chloride	NaCl	7647-14-5	
Niacinamide	$C_6H_6N_2O$	98-92-0	
Sodium benzoate	$C_7H_5NaO_2$	532-32-1	
Sodium hydroxide	NaOH	1310-73-2	
Benzyl alcohol	C_7H_8O	100-51-6	
Various plant and fruit extracts	N/A	N/A	
Hydrogen Peroxide Cream, 20 vol. (6%) containing:			
Water	H_2O	7732-18-5	Hair Care
Hydrogen peroxide	H_2O_2	7722-84-1	Australia
Cetearyl alcohol	$CH_3(CH_2)_nCH_2OH$	67762-27-0	
Sodium laureth sulphate	$CH_3(CH_2)_{11}(OCH_2CH_2)_nOSO_3Na$	9004-82-4	
Disodium phosphate	Na_2HPO_4	7558-79-4	
Salicylic acid	$C_7H_6O_3$	69-72-7	
Phosphoric acid	H_3PO_4	7664-38-2	
Methanol	CH_3OH	67-56-1	Chem-Supply
Milli Q (ultra pure) water	H_2O	7732-18-5	Made daily
n-Butyl chloride	C_4H_9Cl	109-69-3	Sigma-Aldrich

Chemical name	Chemical Formula	CAS No.	Source
Nitric acid	HNO_3	7697-37-2	Flinders Analytical
Olaplex ® No. 1, containing:			
bis-aminopropyl diglycol dimaleate	$C_{10}H_{24}N_2O_3.2(C_4H_4O_4)$	1629579-82-3	Hair Care Australia
water	H_2O	7732-18-5	
phenoxyethanol	$C_8H_{10}O_2$	122-99-6	
sodium benzoate	$C_7H_5NaO_2$	532-32-1	
Olaplex ® No. 2, containing:			
bis-aminopropyl diglycol dimaleate	$C_{10}H_{24}N_2O_3.2(C_4H_4O_4)$	1629579-82-3	Hair Care Australia
water	H_2O	7732-18-5	
Propylene glycol	$C_3H_8O_2$	57-55-6	
Cetearyl alcohol	$CH_3(CH_2)_nCH_2OH$	67762-27-0	
Phenoxyethanol	$C_8H_{10}O_2$	122-99-6	
Glycerine	$C_3H_8O_3$	56-81-5	
sodium benzoate	$C_7H_5NaO_2$	532-32-1	
Olaplex ® No. 3, containing:			
bis-aminopropyl diglycol dimaleate	$C_{10}H_{24}N_2O_3.2(C_4H_4O_4)$	1629579-82-3	Hair Care Australia
water	H_2O	7732-18-5	
Propylene glycol	$C_3H_8O_2$	57-55-6	
Cetearyl alcohol	$CH_3(CH_2)_nCH_2OH$	67762-27-0	
Phenoxyethanol	$C_8H_{10}O_2$	122-99-6	
Glycerine	$C_3H_8O_3$	56-81-5	
sodium benzoate	$C_7H_5NaO_2$	532-32-1	
TCEP	$C_9H_{15}O_6P$	51805-45-9	Sigma-Aldrich
Urea	CH_4N_2O	57-13-6	Sigma-Aldrich

INTERNAL STANDARDS*

Chemical name	Chemical Formula	CAS No.	Internal Standard for:
D5 Amphetamine	$C_9H_8D_5N$	65538-33-2	Amphetamine
D5 MDMA	$C_{11}D_5H_{10}NO_2$	136765-43-0	MDMA
D5 Methamphetamine	$C_{10}H_{10}D5_N$	60124-88-1	Methamphetamine
D3 Morphine	$C_{17}H_{16}D_3NO_3$	67293-88-3	Morphine
D3 Codeine	$C_{18}H_{18}D_3NO_3$	70420-71-2	Codeine
D5 Oxazepam	$C_{15}H_6ClD_5N_2O_2$	65854-78-6	Temazepam, Oxazepam
D5 Diazepam	$C_{16}H_8ClD_5N_2O$	65854-76-4	Nordiazepam, Diazepam
D4 Pethidine	$C_{15}H_{21}NO_2$	53484-73-4	Methadone
D3 Cocaine	$C_{17}H_{18}D_3NO_4$	138704-14-0	Cocaine
D3 MAM	$C_{19}H_{18}D_3NO_4$	136765-25-8	Oxycodone

TARGET ANALYTES (DRUGS)*

Chemical name	Common street or brand names	Drug type	Chemical Formula	Conc. (µg/mL) in DMIX	CAS No.
Amphetamine	Speed, Uppers	Stimulant	$C_9H_{13}N$	1.0	51-64-9
MDMA	Ecstasy, Molly	Stimulant	$C_{11}H_{15}NO_2$	1.0	64057-70-1
Methamphetamine	Meth, Speed, Crystal, Ice	Stimulant	$C_{10}H_{15}N$	1.0	537-46-2
Morphine		Opioid	$C_{17}H_{19}NO_3$	1.0	57-27-2
Codeine		Opioid	$C_{18}H_{21}NO_3$	1.0	76-57-3
Temazepam		Benzodiazepine	$C_{16}H_{13}Cl\ N_2O_2$	1.0	846-50-4
Oxazepam	Serepax	Benzodiazepine	$C_{15}H_{11}Cl\ N_2O_2$	1.0	604-75-1
Nordiazepam		Benzodiazepine	$C_{15}H_{11}Cl\ N_2O$	1.0	1088-11-5
Diazepam	Valium	Benzodiazepine	$C_{16}H_{13}Cl\ N_2O$	1.0	439-14-5
Methadone		Opioid	$C_{21}H_{27}NO$	1.0	125-56-4
Cocaine	Blow, Coke, Crack	Stimulant	$C_{17}H_{21}NO_4$	1.0	53-21-4
Oxycodone	OxyContin, Endone	Opioid	$C_{18}H_{21}NO_4$	1.0	124-90-3

*All drug and internal standard stock solutions provided by Forensic Science SA, Toxicology

Presentation of Work

The work discussed in this thesis has been presented at the following meetings/conferences:

- 2016 - Australia & New Zealand Forensic Science Society (ANZFSS) 23rd International Symposium on the Forensic Sciences (Auckland, New Zealand) - Oral presentation
- 2016 - The International Association of Forensic Toxicologists (TIAFT) and Society of Hair Testing (SoHT) joint meeting (Brisbane) - Oral presentation
- 2015 - RACI Environmental & Analytical Division, 23rd Annual R&D Topics Conference (Melbourne University, Melbourne) - Oral presentation
- 2014 - RACI Environmental & Analytical Division, 22nd Annual R&D Topics Conference (Flinders University, Adelaide) - Poster/3MT (Winner of poster presentations award)
- 2014 - Australia & New Zealand Forensic Science Society (ANZFSS) 22nd International Symposium on the Forensic Sciences (Adelaide) – Poster (Winner of poster presentation award – runner up)
- 2013 - Forensic & Clinical Toxicologists Association (FACTA) conference (Sydney) - Oral presentation
- 2013 - RACI Environmental & Analytical Division, 21st Annual R&D Topics Conference (ANU, Canberra) - Oral presentation

Chapter 1

Introduction & literature review

The analysis of drugs from hair samples can provide forensic toxicologists a great deal of information that cannot be gained through the analysis of blood and urine. This chapter discusses how hair analysis can be beneficial in forensic toxicology, general methods of analysis and some of the issues associated with hair analysis

1.1 Structure, composition and properties of hair

Hair is a very complex matrix and varies from person to person, but contains roughly ~65-95 % protein, 15-35 % water, and 1-9 % lipids, 0.1-5% pigments, and small amounts of trace elements and polysaccharides [1-3]. Human hair contains relatively large amounts of hydrocarbon side-chain amino acids (glycine), hydroxyl side-chain amino acids (threonine), primary amide side-chain amino acids (aspartic and glutamic acid), dibasic amino acids (lysine) as well as disulphide (cysteine) and phenolic amino acids (tyrosine), as well as a variety of lipids including cholesterol, cholesterol esters, free fatty acids and ceramides [1-3]. The types and quantities of the amino acids and lipids can vary depending on the type of hair. For example, there is evidence that curly hair (African hair) has a higher internal lipid content [4, 5]. The diameter of human hair can range from 15-120 μm, and its cross section is made up of three main components of hair: a) outer layer of scale edges or cuticle cells, b) the cortex, and c) the medulla (refer to Figure 1) [1-3].

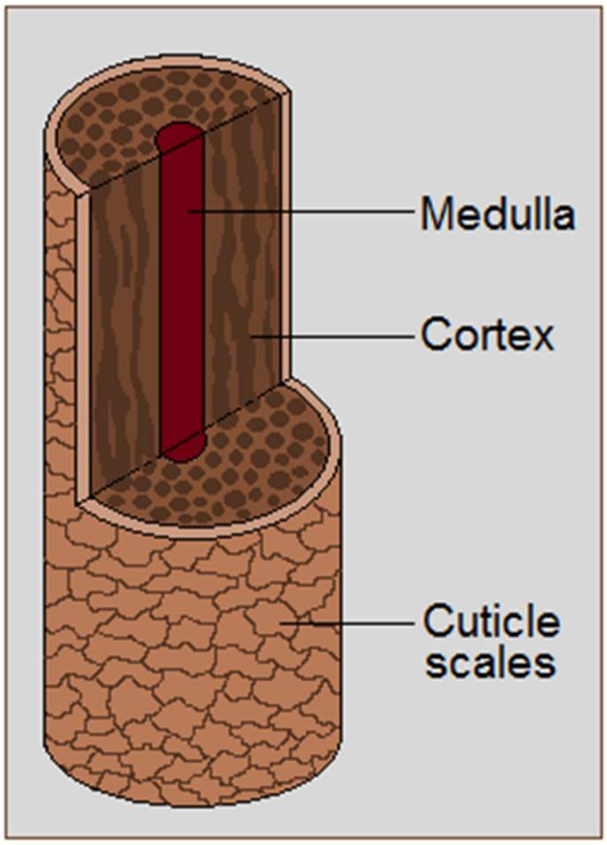

Figure 1: Morphological structure of a hair shaft showing the arrangement of the cuticle scales, cortex and medulla

1.1.1 The cuticle

The outer layer is made up of elongated, overlapping individual cuticle cells approximately 0.5-1 µm thick and 45-60 µm long, and form at 6-7 µm intervals along the hair shaft [1, 2] (refer to Figure 2). Human hair cuticle is generally between 5-10 scales thick [3]. The cuticle's function is to anchor the hair shaft in the follicle and to protect the interior fibres. The cuticle can be damaged by heat and chemicals etc. and hence will become less intact towards the distal end of the shaft (i.e. the tip of the hair) [1, 2]. The cuticle has 4 layers, with the outermost cuticle layer, the epicuticle, being a lipo-protein membrane composed predominantly of sulphur-rich proteins. It and is also generally coated with 18-methyleicosanoic acid (18-MEA), a lipid layer covalently bonded to the surface. This can account for the hydrophobic properties of hair [3, 6]. The layers below, the *A* layer and the exocuticle layer have a high cysteine content, enabling disulphide bonding, and the final layer. The final layer, the endocuticle has low cysteine content [3, 7-9].

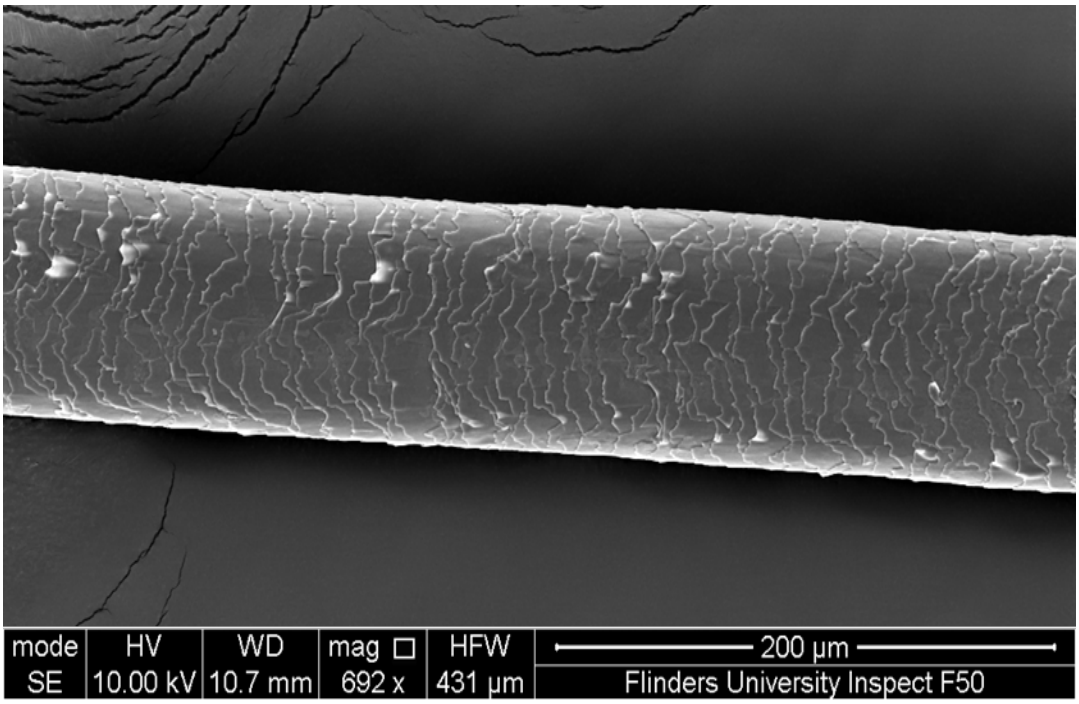

Figure 2: Image of a hair shaft with healthy cuticle scales. Images captured using Inspect FEI F50 SEM

1.1.2 The cortex

The largest component of the hair shaft, known as the cortex, is a hard keratinised structure formed from fibres, ~100 µm in length which takes an α-helical (coiled) form [3]. Keratin is a protein, made up of many sulphur-containing amino acids. Covalent disulphide bonds are formed between the proteins usually hold protein chains together, giving the hard keratinised structure. Located throughout the cortex are air pockets, called fusi. These are small spaces are formed when small portions of fluid are incorporated in the hair at the root during growth. This fluid eventually dries up leaving the holes empty. The cortex also contains the pigment granules which is what give hair its colour. The colour is dependent on the type, quantity and the alignment of pigments. Melanin (from the Greek word *melas* meaning *black*) is the major pigment in hair (and in skin and eyes). Melanin is a polymer made up of the amino acid, tyrosine. The cortex is usually 45-90 µm in width and makes up 90 % of the total hair mass. [1, 3]

1.1.3 Medulla

The medulla is the innermost core of the shaft, which is made up of loosely packed cells and can range from 5-10 µm thick. Thicker hair has more medullar cells, and in thick hair, like beard hair a double medulla may also be observed [1]. The medulla is unique in the way that the thickness not only varies from person to person, but will vary across the hairs on a single individual, or even down the shaft of a single strand of hair. The medulla can be present in full, not at all, or as a segmented medulla [7, 10] (refer to Figure 3).

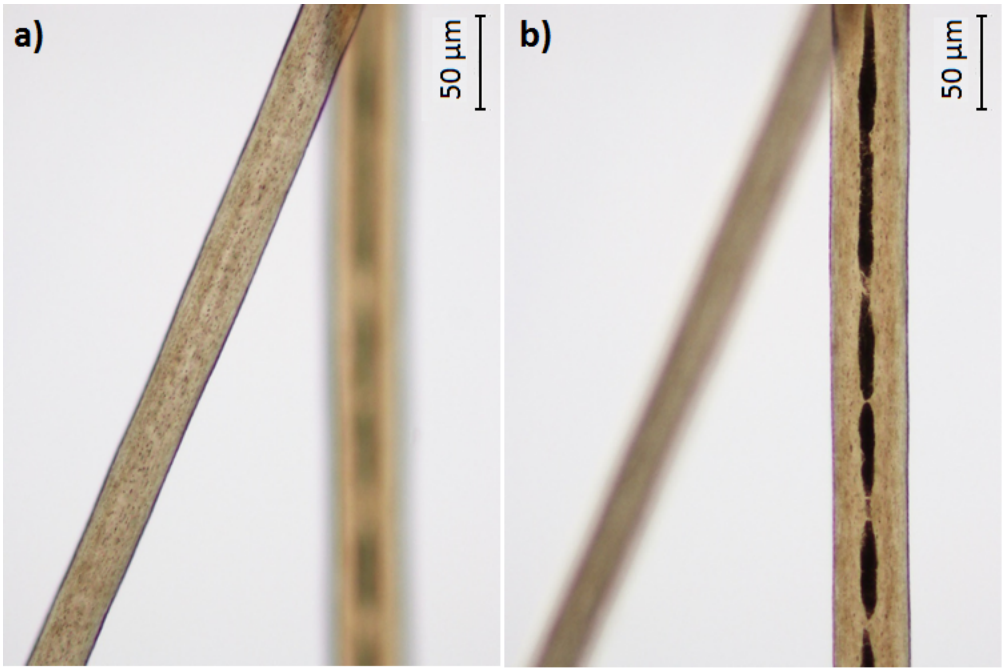

Figure 3: Transmitted light microscope images of two human scalp hair fibres from a single donor. Image a) hair shaft in focus has no medulla, whereas image b) shows a segmented medulla (the darkened sections running through the centre of the hair shaft)

1.1.4 Hair follicle

An average human head contains approximately 100,000 hair follicles (refer to Figure 4), and each will allow for approximately 20 new hair fibres to grow within a lifetime [3]. Hair grows at approximately 1 cm per month, however this can vary (refer to *Rate of hair growth* section) and has three phases of growth; anagen, catagen and telogen phases. The anagen phase is the period during which the hair is actively growing, and will remain in this phase for 2-6 years. On average ~85 % of hairs on the human scalp are in this anagen stage [7]. During the catagen phase growth stops and the follicle/bulb starts to degenerate. This phase will usually last for 2-3 weeks, and is referred to as the transition phase. The final phase, telogen, is the referred to as the resting phase as there is no hair growth. This phase will usually last for approximately 100 days (for scalp hair), following which the hair either falls out then the cycle starts again with a new anagen hair, or the telogen hair is forced out of the follicle when the new hair starts to grown. At any one time, 4-24 % of the hairs on the head are in telogen phase [3, 11].

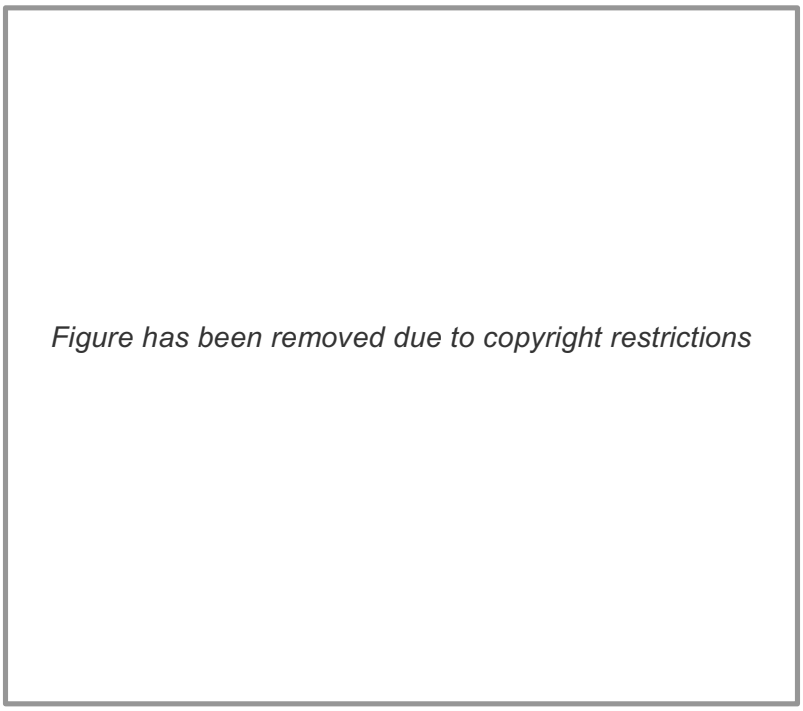

Figure 4: Diagram of a hair follicle, and the surrounding structure. Image sourced from Cooper (2001) [7]

1.2 Hair analysis in forensic toxicology

Hair analysis has many uses in the forensic field, but is most commonly used in forensic toxicology to detect traces of drugs incorporated into the hair. Baumgartner et al. [12] were the first to report the use of hair for toxicological analysis, in 1979, through the use of radioimmunoassay to determine opiate abuse. As hair grows, minute proportion of drugs (and their metabolites) are incorporated into the keratin structure of hair through multiple routes, including via blood capillaries, sweat and sebum [13]. Hair analysis in toxicology is often used in cases involving drug facilitated crimes (DFC's) including drug-facilitated sexual assault, child protection and suspicious deaths. It is also used to look at long term use of drugs, such as the identification chronic poisoning and drug abuse, monitoring drug abstinence such as for drug rehabilitation programmes, and even obtaining a history for victims of drug related deaths. Other, less common potential uses for hair analysis in the forensic field include differentiation between a drug dealer and drug consumer, abuse of drugs in jail, monitoring drug use within a community and detection and identification of personnel exposed to illicit explosives [14-17]. Due to many contributing factors, including contamination (refer to section 1.3.8 Contamination, page 26 and 1.4.5 Axial Diffusion, page 24), a positive result in hair analysis does not necessarily mean the drug was ingested; conversely, due to a negative result is not proof of absence of a drug. Factors that increase the likelihood of getting a false negative include: use of a low dose drug or single use drug (section 1.4.4 Single dose of a drug, page 23), variability in an individual's rate of hair growth (section 1.4.2 Rate of hair growth, page 21), use of cosmetic treatments (section 1.4.1 Cosmetic treatments and environmental influences page 19), and the method in which a sample is collected from an individual (section 1.4.3 Sample Collection, page 22). Consequently, hair analysis is usually done as a complementary technique to blood and urine. For a toxicological analysis that involves blood or urine, there is a limited amount of time after the drug has entered the body in which it can be detected. This is referred to as the 'detection window'. Blood and urine are only able to provide short-term information, due to the limited detection window (hours or days, depending on the drug), whereas detection of drugs in hair is possible weeks, months, or even years, after ingestion and can also provide long-term information about an individual's exposure to a drug (e.g. chronic use vs single dose) [11, 15, 18]. Hair analysis is also less affected by adulterants giving a better idea of an individual's drug use or drug exposure, and if sample is

destroyed/contaminated/questioned/etc. an additional sample can be collected and used for a second analysis. Drugs are very stable in hair, and samples can be stored indefinitely without affecting the concentration of drug in the hair, or affecting the integrity of the sample [15]. The high level of stability of drugs and other compounds in hair has been demonstrated a number of times through the detection of a variety of different analytes; including ethyl glucuronide (EtG, a metabolite of ethanol), cocaine and its metabolite benzoylecgonine, tetrahydrocannabinol (THC) and nicotine; in the hair of mummies, some dating back to 2000 B.C. [19-26]. However, a number of these studies and findings have been criticised due to issues with potential contamination the use of improper analytical techniques, and may not be an accurate confirmation of active consumption [27].

There are currently no recognised standard methodologies for hair analysis, however the Society of Hair testing (SoHT) have published guidelines and recommendations for hair analysis, to assist laboratories with their methodologies and interpretations [15, 28-32].

1.2.1 Segmental analysis and drug monitoring

Segmental analysis (or multi-sectional analysis) is a process whereby a length of hair is cut into sections (usually in 1 cm lengths) along the shaft and analysed separately to measure the drug at different time periods. This type of hair analysis is usually used to discriminate between a single exposure (where the drug of interest is only present in one segment) and long-term use (where the drug of interest is present in multiple segments) [11, 33, 34]. This can help to assess such factors as therapeutic compliance (e.g. to ensure a patient is consistently taking the appropriate dose of drug), drug abstinence (e.g. for workplace/occupational drug testing) [11] and repeated exposures to poisons (e.g. in drug-facilitated crimes to corroborate the victims story that they were drugged and not a regular user) [33, 34]. Segmental analysis can be a useful tool in determining a rough timeline of drug use, however, due to a number of factors discussed later (mainly variability in an individual's rate of hair growth, discussed on page 21, and the various routes which drugs can be incorporated into the hair, discussed on page 25), the localization of drug along the hair shaft is not an accurate representation of the specific day/week a drug was taken. Rather, it can be used as a generalised timeline over a number of weeks or months.

1.2.2 Drug-related deaths

Traditional sample for analysis, like blood and urine, can provide a great deal of information regarding the drug usage of the deceased around the time of death. However, hair analysis can provide information on the weeks or even months leading up to the death. Through segmental analysis toxicologists can determine drug abstinence or, conversely, chronic drug use which can aid in determining the role the drugs played in the death. Similarly, segmental analysis of hair can also be used to determine if acute poisoning has occurred [15].

1.2.3 Drug-Facilitated Crimes (DFCs)

Drug-facilitated crimes (DFCs), crimes in which drugs are used to assist, have become an increasing concern in recent years. Common forms of DFC's include robbery, drug smuggling and homicide, but the most prevalent are drug-facilitated sexual assaults (DFSA). Central Nervous System (CNS) depressants are the types of drugs used in these cases with the most common being benzodiazepines. The pharmacological effects produced by these types of drugs include; confusion, dizziness and disorientation, decreased heart rate, impaired judgment, reduced inhibition, euphoria, lack of muscle control, loss of consciousness and anterograde amnesia (the reduced ability to produce new memories after consuming the drug, making it difficult to recall events). With these crimes, victims may not report the crime for days or even months after the fact, reducing the chance of the usual blood and urine screens yielding a positive result. Hair analysis can be a valuable tool in DFC cases due to its ability to detect drugs long periods of time; i.e. months; after ingestion. The hair is collected approximately 3-4 weeks after the alleged incident, to allow sufficient time for the hair to grow enough for the drug to be far enough along the shaft of hair to enable proper collection [33, 35-38].

A reported case study by Kintz [33] highlights the value of hair testing in drug-facilitated crimes. A 42-year-old man consumed a drink at a party in France, and several hours later noticed his money was missing and had no recollection of the events leading to this point. The concerned victim went to police, but no specimen was collected. Following a string of several similar cases in the same region of France, a judge ordered hair analysis to be conducted for the victims. 7-Aminoflunitazepam, the major metabolite of flunitrazepam (more commonly known as Rohypnol) was detected at 31.7 pg/mg in the corresponding segment of hair, while the distal end tested negative [33].

γ-Hydroxybutyric acid (GHB) is another commonly used drug in DFCs. However, cases involving GHB are difficult due to relatively large concentrations of endogenous compound found in the hair [39]. GHB has been used as an intravenous anaesthetic since the 1960's, GHB is also considered a drug of abuse. Its use in DFC's (DFSA in particular) is due to the alleged enhancement of sexuality, possible abrupt coma-inducing effect, potential amnesia, ease of administration and being readily available. The short half-life of this drug makes it difficult to detect in traditional analysis of blood and urine, where the window of detection GHB is 6 hours and 10 hours, respectively [40, 41]; and hence hair can be used as an additional form of analysis when dealing with DFC's. However, endogenous GHB can be found in the hair at concentrations usually <2 ng/mg but can range up to 12.0 ng/mg. Hence toxicologists must be able to discriminate between endogenous levels and levels indicating exposure or ingestion, which can often be difficult [15, 33, 42].

A case example of where hair was used to detect GHB involved a 19-year-old girl who claimed to have been sexually assaulted after having her non-alcoholic drink spiked. The alleged crime was reported 5 days after incident so it was recommended that the victim wait 1 month so hair analysis could be conducted. An entire length of her hair (8 cm) was analysed for GHB. Through segmental analysis endogenous levels were found to be 0.7 ng/mg. The concentration increased to 2.4 and 2.7 ng/mg at the segments corresponding to the time of the alleged attack, confirming exposure [33].

Another case example involved a 24-year-old female who was sexually assaulted after unknowingly being administered GHB and morphine on numerous occasions during a year-long foreign exchange program. The victim was unaware the assaults had taken place until she was sent video clips in which she is seen being assaulted by a male she met studying abroad. These video clips were not sent to her until arriving back home, at which point the assault was reported. A 20 cm length of hair was collected and segmental analysis was performed on 1 cm segments, equivalent to approximately 1 month per segment. Results determined high levels of GHB (levels higher than endogenous GHB levels for this individual) over a 12-month period corresponding to the time spent abroad. Morphine was also detected in the hair segments over a six-month period, also corresponding to the time spent abroad. Hair segments from the victim, from a time

period not relating the criminal event, was also analysed to ensure the GHB detected was not endogenous [43].

1.2.4 Hair testing for children

Hair analysis can also be used for child protection reasons, i.e. to determine if a child has been exposed to unsafe environments, such as drug-producing homes, or have ingested drugs unsafe for child consumption. Potential sources of exposure in infants and children include [15]:

- Mothers who abuse drugs and alcohol during pregnancy – hair collected from newborn,
- Drugs are also transferred in maternal milk to babies who are breastfed,
- Mobile young children who can access drugs, or come in contact with contaminated surfaces,
- Smoke in the atmosphere,
- Sweat from close contact with adults or siblings who use drugs

A number of case studies have detected drugs in children's hair who have had prolonged exposure to drug contaminated environments. Moller *et al.* [44] reported the examination of the hair of 75 children (at an average age of 6.5 years) who had recently been removed from homes where marijuana was grown or other drug operations had taken place. Of the 75 hair samples analysed, 32 % (24 samples) tested positive for illicit substances. Similarly, Farst *et al.* [45] examined hair from 107 children all under the age of 12, who were suspected to have been exposed to the manufacture of methamphetamine by their caregivers; however only 103 children had hair specimens suitable for analysis. Of these 45 % of the children tested positive for methamphetamine alone and 15 % tested positive for both methamphetamine and amphetamine (likely present as a metabolite of methamphetamine).

Chatterton *et al.* [46] reported the investigation of a deceased 14-month-old child, who was found in their cot unresponsive and not breathing. A toxicology analysis revealed a lethal concentration of methadone in their blood, as well as the presence of EDDP (metabolite of methadone), chlorpheniramine and diphenhydramine. Hair samples were collected from the deceased child and their sibling to determine if the children had

previous exposure to methadone. The results from the segmental hair analysis indicated presence of both methadone and EDDP along the entire length of the hair for both children (sibling presented lower concentrations), suggesting ingestion of methadone on more than one occasion. However, even though the results point to ingestion, the analyst cannot prove that the parents knowingly administered the drug, and hence the author suggested poor housekeeping as a possible cause of accidental ingestion. The number of times the child was exposed to methadone, prior to over-dosing, could also not be substantiated.

Neonatal hair can also provide information of *in utero* drug exposure. Studies have determined that scalp hair present at birth can provide information of drug exposure from approximately the 28th week of prenatal development, i.e. during the third trimester, and samples can be collected at any point during the first 3 months of life [47-51]. A case study involving an infant, exhibiting signs of neonatal withdraw syndrome, whose mother was suspected of ketamine abuse during pregnancy found a significant amount of ketamine and its metabolite norketamine present in the neonatal hair samples (2 cm long, 25 mg) from the infant. Even though the mother denied the use of ketamine, these results confirm fetal drug exposure and were able to aid the assessment and treatment of the neonatal withdraw syndrome [52].

1.2.5 Heavy metals in hair

Monitoring heavy metal exposure in humans is another important aspect in forensic toxicology. It is well known that exposure to some heavy metals and metalloids, such as mercury, lead and arsenic, can exert toxic effects in humans. Human exposure from pollution of heavy metals is on the rise due to the continual use of activities such as mining, use of harmful fertilizers, and combustion of fossil fuels [53, 54]. Particular concerns arise for people living near factories that expel large amounts of heavy metal waste, either through gas emissions into the atmosphere, or through waste disposal [55]. Detection of heavy metals can be monitored in environmental samples such as soil, drinking water, food sources and waste water [55, 56], as well as biological samples, such as blood, urine and hair [57, 58]. For hair analysis, sample preparation usually involves digestion in heated acid (usually nitic acid), or dry ashing (i.e. heating to very high temperatures to destroy and remove the organic material), followed by analysis via ICP-MS [54, 58, 59].

1.3 Sample preparation and analysis

A general procedure of hair analysis usually consists of a wash/decontamination step, digestion, either through solvent digestion, acidic or basic digestion, or even enzymatic, followed by a clean-up step, usually LLE or SPE, and finally analysis, by immunoassay, LC-MS or GC-MS.

1.3.1 Sample Collection

An advantage of hair analysis is that specimens can be collected with less embarrassment and it is less invasive, compared to urine and blood sampling [33]. Samples should be approximately a pencil thickness lock of hair, cut from the posterior vertex or occipital regions of the scalp (refer to Figure 5) [11, 15]. If this is not a viable location collection of smaller hair samples from multiple sites is acceptable. However, the vertex posterior (crown) is used in preference to other areas on the head due to this area having less variability in hair growth rate, the number of hairs in the growing phase (anagen) being more constant, and the hair in this area is less subject to age-related and sex-related influences [33].

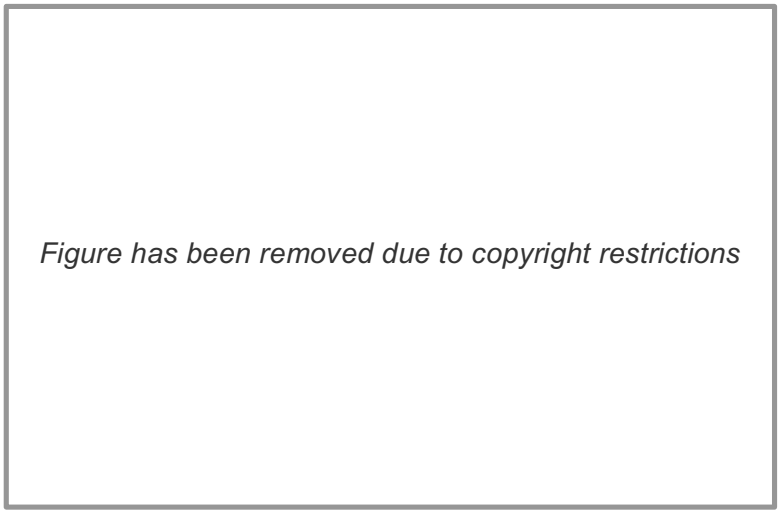

Figure 5: Ideal regions of the scalp for hair sample collection. Image sourced from LeBeau *et al.* [11]

Scalp hair in general is the preferred sample for hair analysis as it has the most consistent growth rate (refer to *Rate of hair growth* section), as collection of sample is also a lot less

invasive which means the specimens can be collected with less embarrassment compared to urine and blood samples and can be done under strict supervision (e.g. of a law enforcement officer) to prevent adulteration or substitution [14, 33]. However, if unavailable other sources of hair can be used, including pubic, axillary (armpit), and beard hair. Studies have determined there is good correlation between drug concentrations in the scalp hair and the concentrations in hairs from other parts of the body making them a suitable alternative to scalp hair to prove previous drug use. However, concentrations tend to be higher in pubic and axillary hair than in head hair, and hence it should be noted that higher quantitative results in pubic hair do not necessarily represent heavier drug use [60-63]. It is suggested this may be due to better blood circulation, a greater number of apocrine glands, different growth rates, and a different telogen/anagen ratio at the axillary and pubic regions [14, 62]. Other issues arise with using hair from these alternative locations including the growth rates and dormancy characteristics from these sites differ from head hair [15, 64].

The samples should be cut as close to the scalp (or skin) as possible, and the location of the sample specified. The root and distal (tip) ends if the sample should also be marked [14, 15, 33]. Once the sample has been collected, it can be stored indefinitely in the right conditions. Hair samples stored in the refrigerator or freezer or in an environment with moisture will swell resulting in some drug loss. Hence, samples should be stored in a dry, dark environment at room temperature. Hair samples should also be stored in aluminium foil, an envelope or plastic tube [14, 15]. When collecting hair for single hair analysis, it is vital to ensure that the hair is in its anagen phase, otherwise the drugs could have been ingested much earlier than analysis would suggest. When hairs are cut it is impossible to determine the growth phase as the root will be absent. Hence, hairs should be plucked and not cut for single hair analysis. Fortunately, collection of a bundle of hairs, for homogenisation/extraction, only a small fraction of hairs will be in the telogen phase and will not greatly affect the analysis.

Special considerations are required for cases involving DFC's and other cases involving a single dose when sampling hair. These hair samples should be collected a minimum of 4-6 weeks after the alleged incident/ingestion to allow sufficient time for the drug to be incorporated into the hair. If the analysis returns positive, it is also recommended that a second sample be taken to confirm results. The complainant must not have any cosmetic

treatments or have hair cut in between sample collections. This is important as DFC's usually involve a single dose which can be difficult to detect, and cosmetic treatments such as colouring, can have a detrimental effect on the drug making detection even more difficult [15].

1.3.2 Washing

Washing of the hair samples needs to be conducted prior to analysis to remove any hair products (e.g. hair gel), sweat, sebum and other surface material (including skin cells, head lice, etc.), and to remove potential external contamination of drugs from the environment [15]. There is no uniformity or consensus for appropriate wash protocols between laboratories, and as such a variety of different agents have been used, including detergents and shampoos, surgical scrubbing solutions, surfactants and organic solvents (e.g. acetone, diethyl ether, methanol, ethanol, dichloromethane, hexane or pentane). Wash procedures even vary on the volume of washing agent used, how long, and the repetitions [65]. However, there is general consensus that organic solvents such as dichloromethane or acetone will only remove contamination from the surface, whereas aqueous solutions will swell the hair and extract the drugs from the hair sample, potentially resulting in a false negative. The final wash is usually collected and analysed along with the extracted hair sample to ensure no, or minimal, contamination. It should be noted that a wash procedure that will effectively remove all traces of external contamination without removing the drugs that are incorporated into the hair has not yet been identified. In all cases the possibility of external contamination influencing the analysis should be taken into consideration when interpreting the results [15].

1.3.3 Homogenisation, extraction and clean-up

Hair is not a homogenous matrix, and hence prior to analysis hair samples must undergo steps to ensure homogeneity. There are currently two main methods used to homogenise hair samples: pulverisation and segmentation (cutting into small pieces). As well as homogenising the hair, this will increase the potential for better drug extraction as the surface area is increased. This means, in general, ball-milling/pulverisation will result in better extraction than cutting. A study done by Mönch *et al.* [66] indicated that the use of pulverisation via ball-milling prior to extraction resulted in significantly higher levels of ethyl glucuronide (EtG) when compared to standard cutting methods. However, ball-

milling/pulverisation tends to require more sample, and result in significant loss of sample. It can also be difficult when preparing large batches of samples as the ball-milling equipment requires extensive cleaning between each sample [7].

Following homogenisation an extraction procedure is carried out, either through solvent extraction, acid or base digestion, or even enzymatic digestion. This where samples are incubated for a known time and temperature, usually in methanol, acid, sodium hydroxide or buffer, and is done to release the target analytes from the matrix [14]. However digestion with sodium hydroxide (or under acidic conditions) may result hydrolysis of some drugs and metabolites [67]. Like with the washing, digestion procedures vary on the type of solvent, the volume, temperature of incubation and the length of incubation. For example, methanol extraction has been reported to take 5-18 hours in a sonication bath [68]. Microwave energy has recently been used as an alternative to overnight/lengthy incubations to reduce the time required for the extraction. MAE has been used to detect a number of drugs in a variety of drugs classes including anaesthetics [143], central nervous system (CNS) depressants [142], opioids [144-147], analgesics [153], benzodiazepines [151] and tricyclic antidepressants [148] from various biological matrices. However, there are only a handful of reports on the extraction from hair [67, 137, 141, 149]. This type of extraction technique is investigated further in Chapter 3.

Other forms of digestion that have been reported include the use of the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP) [69], and dithiothreitol to break up the longitudinal chains of the keratin protein of the hair by reducing the disulphide bonds (usually done in conjunction with an enzyme, Proteinase K, in the case of dithiothreitol) [64]. Relating to this, is the possible use of ionic liquids for digestion and extraction. While this has not yet been investigated for the use in forensic toxicology, ionic liquids are a very versatile type of chemical that is currently used in a wide variety of ways in a number of fields, including analytical chemistry. This is discussed further in Chapter 4.

Exhaustive extraction, using a Soxhlet apparatus, could also be used for drug extraction. However, this is more commonly used for extracting lipids for hair, rather than drugs [70]. Additionally, extraction techniques such as these require a large amount of solvent and sample, and take a considerable amount of time and effort. As a result, exhaustive

extraction techniques are generally not suitable for a small scales analysis like hair analysis for drug detection.

Following the digestion/extraction, the final step in the sample preparation is the clean-up. This can be conducted either through solid-phase extraction (SPE), liquid-liquid extraction (LLE) or by filtration. Digestion/extraction straight into an organic solvent, such as methanol, will usually only require filtration or solvent transfer. To get the sample ready for analysis, the organic solvent is evaporated down and reconstituted in the appropriate solvent.

Additionally, there have been reports of pulverized-extraction methods that involve ball-milling with the hair sample and extraction solvent all in one tube [71, 72]. This not only removes the need for incubation time, but also reduces sample clean up.

1.3.4 Instrumentation and analysis

For hair analysis, a true "general unknown" screening does not exist and must rely on targeted screening methods. Generally, immunoassays and GC-MS/MS methods are used for screening, but are limited to the number of target analytes due to the decreasing sensitivity with the increase in target analytes. Similar to urine analysis, immunoassay is used as a general screening technique for hair analysis [15]. However, unlike immunochemical screening for urine analysis, in which it is used to detected metabolites rather than the parent drug, in hair analysis the parent drug is present in higher concentrations than the metabolites (if the metabolites are present at all). And hence hair analysis requires a different kit to the urine screen; one with a relatively high cross reaction of the parent drug rather than the metabolite [73]. As well as immunoassay, many laboratories have employed LC-MS for general screens. Due to its sensitivity and selectivity capabilities LC-MS/MS has the potential to screen for a greater number of analytes due to the high sensitivity of the instrument [73, 74]. For confirmation, a technique that has sufficient sensitivity for low drug levels is required, as hair samples contain much lower concentrations than the traditional blood and urine samples, as well as a great level of specificity. Hence LC-MS is usually used as the confirmation technique [15, 74].

1.3.5 Single hair analysis

Single hair assessment is another potential method of analysis, with techniques such as matrix-assisted laser desorption/ionization (MALDI) and Infrared microscopy already being used. Single hair analysis provides an effective method of analysis, with minimal sample preparation. However, due to the nature of single hair analysis, it has not been implemented for routine casework and screens, as batch analysis is not practical and would be too time consuming and labour intensive.

Matrix-assisted laser desorption/ionization (MALDI)

A number of studies using matrix-assisted laser desorption/ionization (MALDI) or MALDI coupled with mass spectrometry (MALDI-MS) for drug detection in hair have been reported [75-86]. MALDI methods provide advantage over traditional screening methods such as LC-MS and GC-MS methods, as it allows for a direct analysis of the hair itself to not only determine the presence of a drugs or metabolite but to determine the distribution along the hair. Beasley *et al.* [87] reported a MALDI-MS method that could simultaneously detect THC and metabolites in a single workup and analysis. This shows an additional advantage over traditional LC-MS and GC-MS techniques as the main metabolite of THC is a THC-glucuronide conjugate which is generally not detected using those techniques without extensive sample workup. However, some MALDI-MS methods, while being a faster alternative to LC-MS and GC-MS, still require sample preparation procedures including extraction and segmentation. Prior to mounting the hair, it is common for the hair to be longitudinally cut or microtomed which can be difficult and cumbersome without the appropriate equipment. Some methods have also described using the hair extract, rather than the hair itself for MALDI-MS analysis.

Infrared Microscopy

Infrared microscopy is not a technique commonly used in the detection of drugs in hair, but has the potential to examine the interior of the hair without being affected by any external contamination. This method is beneficial as it is able to differentiate contamination on the exterior surface from drugs absorbed into the hair from ingestion. A single strand of hair is microtomed either cross-sectionally or laterally, which allows for IR spectra to be obtained of the cortex and medulla. Infrared functional group imaging is then obtained across the hair to determine the presence of drugs. This method is able to differentiate between contamination and drug that has been ingested, as well as locating

the position of the drug along the hair, and how the drug was incorporated, i.e. whether the drug was incorporated into the medulla, the cortex or the cuticle. Quantification of the drug by this method, however, is not as accurate as the traditional methods of hair analysis (i.e. extraction followed by GC/MS or LC/MS), and therefore these techniques should be continued to be used. As such a small portion of hair is used, infrared microscopy is also only useful for detecting chronic use of drugs. The final issue associated with this analytical technique is that it is very labour intensive and requires extensive sample preparation [88, 89].

1.4 Pitfalls in hair analysis

Even though hair analysis is a useful tool for detecting traces of drug and analyte, it has a number of downfalls. The use of cosmetic hair treatments and constant exposure to natural factors, e.g. sunlight, weather, water, pollution, etc. can cause degradation of the target analyte. Error in sampling procedures and varying growth rates of hair can result in false negative or inaccurate interpretations of dosing timelines, and the various routes of incorporation of drug into the hair and contamination also makes interpretation of results difficult.

1.4.1 Cosmetic treatments and environmental influences

Cosmetic hair treatments, such as bleaching and colouring, cause damage to the keratin structure, and use of cosmetic products e.g. gel and hair spray can interfere with analysis and potentially result in false-positives for fatty acid ethyl esters (FAEE) indicating alcohol consumption, when products with high ethanol content are used [90]. Chemical straightening, which has a similar process to perming, has been found to reduce the concentration of cocaine, and its metabolites benzoylecgonine and cocaethylene, phencyclidine and tetrahydrocannabinol, by 33-94 % for standard reference material hair, and 70-95 % for authentic drug hairs [91]. Thermal straightening has been shown to result in decrease in concentration of parent drug while simultaneously increasing the concentrations of metabolites [92, 93]. On a few occasions, the parent drug was also seen to increase which may be due to denaturation of the hair matrix by thermal treatment possibly causing a better extraction of the drug. Additionally, Blank and Kidwell's studies on external contamination demonstrated that hair treated by dyeing incorporated externally applied drugs [94].

Even day to day treatments can damage and alter the structure of hair, which may result in drug loss. As well as the strong crosslinking disulphide bonds, hair also contains other weaker bonds that also link the polypeptide chains such as Van der Waals interactions, hydrogen bonds, and coulombic interactions referred to as salt links. These weaker bonds result mostly from the attraction of positively and negatively charged groups, or partial charges. They can be easily broken just by wetting the hair, and reconnected simply by styling wet hair using a brush or curlers, or by using heat treatments such as straightening or curling. However, these affects are only temporary. Hair will gradually absorb moisture from the air or will be washed again, allowing the hydrogen bonds to break and reconnect [95]. And while some studies have also shown a concentration decrease due to repeated washing [96] other studies have shown that repeated shampooing has no effect on drug concentration, even the 'Ultra Clean' shampoo marketed to remove drugs only slightly reduces drug concentration but not enough to drop below the limit of detection [97, 98].

A new treatment on the market, Olaplex®, is a widely used restorative treatment that also has the potential to interfere with hair analysis. The Olaplex® treatment, usually incorporated into bleaching treatments, is marketed as a bond multiplier, which claims to repair and rebuild broken crosslinking disulphide bonds [99, 100]. There is currently no literature on how this treatment may effect hair analysis. However, there is some potential that the use of Olaplex® could degrade drugs in hair, similar to the treatments mentioned above. Additionally, the compounds introduced into the hair during the Olaplex® treatment could perhaps inhibit extraction. It is important that the forensic analyst conducting hair analysis is aware of any cosmetic treatments the hair has had and the potential effects of these treatments of the analysis and interpretation of the results. Investigations into the effect of Olaplex® on hair analysis is discussed further in Chapter 5.

A negative hair result does not necessarily mean that no drug was taken, the analytical procedure may not being sensitive enough, and should be interpreted accordingly [18]. The Society of Hair Testing consensus on testing for doping agents states that in the case of a positive urine analysis result, a negative hair result cannot overrule the positive urine result [101]. Unlike in blood samples, the concentrations of the target analytes in hair cannot be correlated with a dose, nor can they be used to pinpoint an exact date of administration, which will also need to be considered when interpreting results [11].

1.4.2 Rate of hair growth

Human head hair grows at an average rate of 1 cm each month [7, 11, 15], and for the purpose of hair analysis this is used as the rule of thumb. However, the reported growth rates are actually incredibly varied, with the slowest reported growth rate at 0.6 cm per month and the fastest at 3.36 cm per month [1, 7]. There are also many factors that affect growth rate including age, gender, pregnancy and hormones, and even seasonal effects and pigmentation of the hair [11]. A study by Myers *et al.* [102] demonstrated that female hair grows faster (at 0.36 mm/day) compared to males hair (at 0.34 mm/day). Similarly, the growth rate between age groups also varied, with ages 9-11 years had an average growth rate of 0.31-0.41 mm/day, compared to 0.30-0.35 for 21-30 years age group, 0.34-0.36 mm/day for 64-74 years, and 0.30 mm/day for 75-84 years. Van Neste [103] also demonstrated in a study in hair growth in 24 women, that pigmented hair grows at an average rate of 0.34-0.37 mm/day, compared to un-pigmented hair (white hair) at an average rate of 0.37-0.40 mm/day. The growth rate also differs for hair on different parts of the body (refer to Table 1).

Table 1: Reported growth rates of hair across the body [1, 7, 11, 102-109]

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Another issue associated with the growth rate of hair is that it can take 7-10 days for growing hair to reach the surface of the scalp, and hence, hair cut from the scalp does not necessarily represent recent hair growth [7]. It should be noted that growth rates for children and infants may vary considerably from those applicable to adults, and as such

the average hair growth rates which apply to adults should not be assumed for infants and children [15].

1.4.3 Sample Collection

Hair samples are usually collected posterior vertex or occipital regions of the scalp by securing a lock of hair, approximately the thickness of a pencil, with a rubber band, twist tie or foil, and cutting as close to the scalp as possible. It is important to cut the hair as close to the scalp as possible to ensure the best possible chance of collecting the section of hair with the drug of interest, particularly if it after a single exposure, and to allow for appropriation timeline estimations for segmental analysis. However, issues arise during sample collection, especially when evidence collector is inexperienced. These include, either the lock being cut too far away from the scalp, leaving too much hair left on the scalp, or uneven sampling, which will result in uneven amounts of hair left of the scalp. The shape of the bundle, i.e. if a cone shape is formed; and the angle of the scissors during sample collections can both result in uneven sampling (refer to Figure 6). For this reason, some institutes, like Forensic Science South Australia, advise against securing or tying the lock of hair prior to cutting in an effort to avoid the coning effect. Instead it is recommended to gently pinch the hair and cut as close to the scalp with the scissors flat to the head and use the foil to secure the cut hair in place.

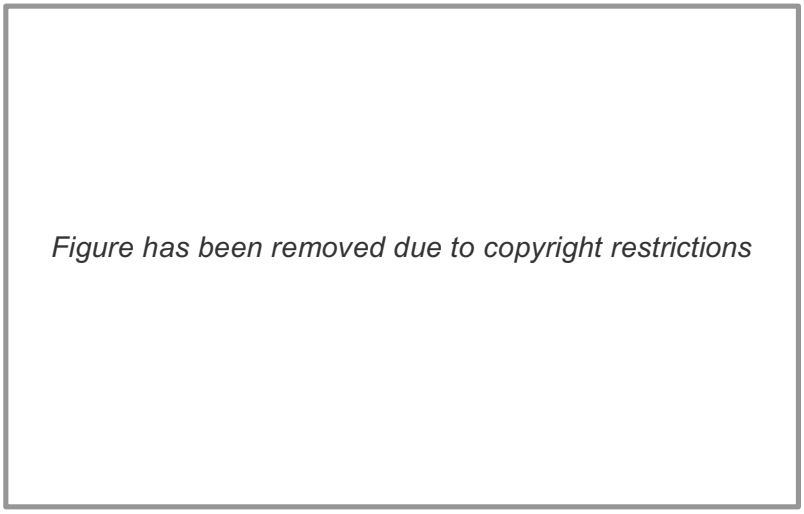


Figure 6: Diagrams of a) a bundle of hair ready to be cut, depicting how the hairs form a cone-shape as it is secured in a bundle which will result in uneven sampling, and b) the hair left on the scalp after sampling depicting angle of the scissors will also affect the amount of hair remaining on the scalp [11]

A study was conducted by LeBeau *et al.* [11] whereby fourteen volunteers (five experienced evidence collectors, nine novices) were instructed to cut hair samples from *Barbie Styling Head dolls* to simulate collection of a real hair sample. Eight dolls were set up with hair lengths ranging from 3-20 cm, and volunteers were asked to cut samples from two dolls (one with long hair, one with short hair). Prior to sampling each volunteer was given the exact same written and verbal instructions, with an emphasis on the importance of cutting the sample as close to the scalp as possible. A single individual then measured the length of hair (to the nearest millimetre) left on the scalp, longest and shortest length, after sampling (refer to Figure 6b). This study was to demonstrate how each person's sampling technique differs and how this could affect analysis and interpretation of data. The result of this study show that hair sampling is not uniform, making interpretation of results difficult, with the average lengths of hair being left on the head 0.45-1.4 cm for long hair collection, and 0.7-0.9 cm for short hair collection. The longest length of hair left on the scalp was as high as 3 cm, suggesting that 3 months of data could be missed using this sample of hair. Hence, the authors have suggested that hair collection should take place 8 weeks after the ingestion, in particular with hair analysis in DFC's, rather than recommended 4 weeks to ensure the sample collected fully represents the exposed period [11].

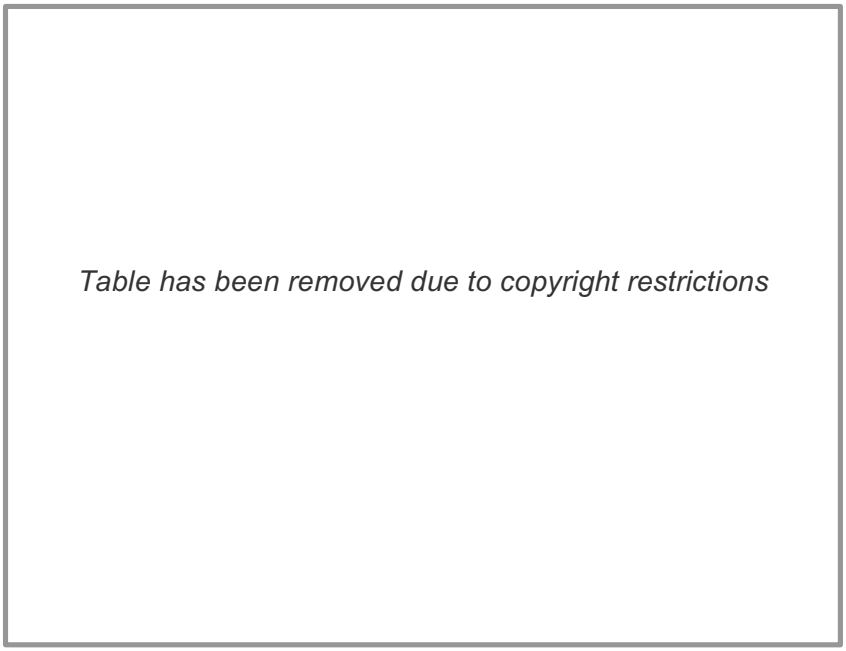
1.4.4 Single dose of a drug

A negative hair result does not necessarily mean that no drug was taken. It could be a result of the analytical procedure not being sensitive enough, and hence, should be interpreted accordingly. This is very common in the case of a single dose as the concentration in the hair can be too low to detect or quantify [18]. Since there is currently no recognised standard method for hair analysis, suggested cut off concentrations have been published (refer to Table 2) to assisted in appropriate detection and quantification.

Previous studies have already revealed there are some drugs in particular that are not detectable in the hair after a single exposure. Segura *et al.* [110] could not detect any testosterone esters after single doses of testosterone enanthate (250 mg, intramuscular administration), testosterone propionate (25 mg, intramuscular administration), or testosterone propionate (120 mg, oral administration). Similarly, it has also been found that both nandrolone decanoate and nandrolone could not be detected after a single dose of nandrolone decanoate [18, 111]. These molecules are quite large, and a potential

reason why these drugs in particular are not being detected could be that the drug is not actually being incorporated into the hair, due to their large size, preventing them from being transferred from the blood capillaries to the hair shaft. In terms of drugs of abuse and drugs commonly used in DFC's a number of studies have been able to detect target analytes after a single dose. Villain *et al.* [112, 113] detected bromazepam in the proximal hair segment of two individuals one month after a single 6 mg dose, and zolpidem in the hair of 2 individuals 3-5 weeks after a single 10 mg dose. Xiang *et al.* [34] detected estazolam in the proximal hair segments of fourteen individuals after a single dose, ranging from 1-6 mg. However, there are numerous drugs, including heroin, morphine, amphetamines and cannabis, that have not yet been assessed in terms of detection in hair after only a single dose, which is an issue when attempting to interpret results [18].

Table 2: Recommended cut-offs for substances and metabolites in hair to identify use [15, 73]



1.4.5 Axial Diffusion

Axial diffusion is also a factor that should be considered when differentiating between regular use and a single dose, and determining a timeline for drug exposure. Through segmental analysis, administration of a single dose of a drug could be confirmed by the presence of the drug in the proximal segment (root) and its absence in the other segments. However, there have been reports of a drug being detected in 2 or 3 consecutive segments after a single exposure [34, 112, 114-118]. Kintz [114] has outlined

a number of possible explanations for this phenomenon occurring, including growth rate of hair, hair strands within the sample being in different growth phases (anagen, catagen or telogen), external contamination, contamination and additional incorporation through sweat, and changes to hair structure or analyte concentration due to cosmetic treatments. Single hair analysis and drug mapping may be a way to avoid misinterpretation of results when axial diffusion has occurred.

1.4.6 Incorporation of drug into hair

Hair is not a homogenous matrix and drugs are incorporated into the hair via multiple routes and tend to bind to the functionalities in proteins through van der Waals interactions. The incorporation into the hair can occur from blood capillaries at the root bulb, sweat, sebum and external contamination. It has not yet been determined to what extent each of these routes contributes to the overall concentration of drugs in hair, and hence should all be taken into account when interpreting findings [7, 11, 15, 18, 119, 120]. However, the main route is thought to be incorporation into the growing fibre by passive transfer from blood into the matrix cells and melanocytes to become entrapped during keratogenesis.

Melanin has been shown to play a large role in the degree of incorporation. A study conducted in bulls showed that in black hair the concentration of testosterone was four times higher than other colours, suggesting that pigmentation is a strong influence on the incorporation of some drugs [69]. Codeine is another known drug where concentration in hair after an oral dose is primarily dependent on melanin content, i.e. higher concentration of melanin will result in higher concentration of codeine in the hair. Melanin content, and melanin affinity, is not the only factor that determines if a drug is successfully incorporated into hair as drugs have been detected in hair of albino animals which lack melanin [14]. The degree of incorporation of drugs into the hair also depends on chemical structure and properties of drug (e.g. melanin affinity, as well as lipophilicity and membrane permeability) [13, 18]. The following properties have been found to lead to good incorporation of the drug in hair [18]:

- Presence of a nitrogen atom (binds with melanin)
- Absence of an acidic functionality

- Basic drugs (better penetrating ability to break through the cell membrane)
- Lipophilic (greater ability to pass through the cell membranes)
- Presence of long N-alkyl and N-phenyl functionalities

1.4.7 Contamination

As mentioned before, a negative hair result does not necessarily mean that no drug was taken. Conversely, a positive may not always mean the drug was taken. External contamination is a significant problem in hair analysis, and can occur from being within the same environment as someone smoking a drug, handling a drug then touching the hair, or even coming in contact with a contaminated surface and then touching hair [7]. Washing the hair prior to extraction is often done to remove any contamination. However, it is still not clear how effective these washing procedures are at removing all traces of contamination. A recent study in 2016 by Cuypers *et al.* [121] on the consequences of decontamination procedures, has demonstrated that a number of commonly used washing solvents, including dichloromethane, isopropanol, acetonitrile and dichloromethane/water, were ineffective at removing externally contaminated cocaine from hair samples. Additionally, while water and methanol were able to remove some of the externally contaminated cocaine they also spread the cocaine further along the shaft of hair. Other studies suggest that in some cases it is not possible to differentiate between contamination and active use, even after a single exposure [122] (also refer to *Cocaine contaminated hair* section). Conversely, there is even evidence that the wash may be removing target analytes that were ingested. Hence, external contamination can result in a false positives, and washing to remove contamination can result in a false negative [89].

To differentiate between systemic exposure and external contamination, one proposed method is to compare the concentration of drug residue in the wash with the concentration in in the hair. According to some studies, the concentration of drug in the hair after washing should exceed the concentration in the last wash by at least 10 times [97, 123]. However, Blank and Kidwell [94] found that applying this discrimination technique to hair samples taken from a crack smoker and an intravenous (IV) cocaine user, and externally introducing *ρ*-bromococaine (i.e. the contaminant), it was found that the *ρ*-bromococaine could successfully be confirmed as a contaminant for the IV cocaine

user, but the 'crack' smoker would have been considered a user of *ρ*-bromococaine if this criterion was used. Additionally, the IV cocaine user would not have been considered a cocaine user under this criterion, as the ratio of the cocaine in the hair to the final wash was 7.6:1. Another proposed method of discriminating between contamination and a legitimate drug usage is parent drug/metabolite ratios. If a metabolite of the drug of interest is also present in sufficient quantities then a contamination can potentially be ruled out. For example, a benzoylecgonine/cocaine ratio < 0.05 suggests external cocaine contamination and is recommended to report a negative cocaine hair [124].

Contamination can also be beneficial in some situations, as it can be used to gain information on the environment, for example in the case of child protection. Coming in contact with a person taking or who has recently taken drugs can potential result in contamination via transference of sweat, touching contaminated clothes, etc. Hair analysis studies of children of cocaine users have shown positive detection for cocaine, and in some cases its metabolite benzoylecgonine, resulting from passive exposure. Positive results from exposure provides an evidentiary basis for the removal of children from dangerous households [125, 126].

Contamination of post-mortem samples

Post-mortem samples have been found to be an extra challenge when it comes to contamination due to potential long term contact with organic and putrefied materials containing drugs. Studies by Kintz [65] verified that exposure to organic material containing drug can produce a false positive. Hair samples free of flunitrazepam and its metabolite, 7-aminoflunitrazepam, were incubated in 10 mL whole blood, spiked with 0.1 µg/mL 7-aminoflunitrazepam (an acceptable concentration of the metabolite for a single dose of flunitrazepam). Irrespective of length of time and temperature at which the hair was left in contact with the contaminated fluid, the spiked hair tested positive for 7-aminoflunitrazepam. Detection of both parent drug and metabolite(s) is often used as a method of indicating whether a sample is contaminated or not. If the metabolites are present it suggests drug consumption, whereas absence of metabolites could indicate contamination. However, this method cannot always be used for post-mortem samples, as both the parent drug and metabolite(s) may be present in biological, putrefactive tissues in contact with the hair. This shows that post-mortem hair analysis of a single segment of hair is unable to differentiate between actual drug use and contamination

due to contact with biological specimens, and unable to differentiate between a single dose and chronic use. For this reason, the authors suggest a single segment of hair should not be used to discriminate long-term exposure to a drug. Homogenous results throughout the hair shaft can indicate contamination and hence analysts are also encouraged to perform multi-sectional analyses. This issue with segmental analysis for post-mortem hair was demonstrated by Beyer *et al.* [127]. For a drug undergoing regular ingestion, e.g. daily use, the drug concentrations in hair generally decrease from root to distal end of the hair, due to natural "wash-out" of the drugs [15]. However, Beyer *et al.* found an exponential increase of 6-monoacetylmorphine and acetylcodeine concentration from root to tip in a post-mortem case. This was believed to be the result of external contamination [127].

Cocaine contaminated hair

Contamination of hair with cocaine has become a particular concern as it has been found that cocaine may be absorbed into hair via external contamination, which results in false positives [128, 129]. This has been demonstrated in studies by Strout *et al.* [119] and Romano *et al.* [130] in which locks of blank hair were manually contaminated with cocaine hydrochloride. Following the contamination process the hair underwent hygienic treatments (i.e. shampooing) to mimic real-life conditions. Small samples of each lock were taken and analysed at different time periods over a 10-week period. Each study assessed multiple decontamination processes, but found there was still sufficient levels of cocaine present in the hair to be considered a false positive. Metabolites of cocaine were also looked for in the hair samples, and it was found that benzoylecgonine was also detectable over the 10 weeks period in both studies. As benzoylecgonine was not present prior to contamination and was not spiked to the hair, it was formed in situ. It is thought this may be occurring due to hydrolysis or other non-metabolic processes of cocaine deposited in hair [119, 131]. However, it has been noted by some other authors that the concentration at which the cocaine hydrochloride was applied to the hair samples was rather high, and has been questioned as to whether this is a realistic representation at which an individual can be contaminated [129, 132]. It has even been suggested that the wash may not have been extensive and rigorous enough, and may be beneficial to reassess the decontamination procedures used [132].

1.4.8 Reference material

When it comes to quality control and quality assurance, as well as method development, validation and accreditation, getting hair with known concentrations of drug can be quite difficult and expensive. When using blood and urine samples, drug stocks can be spiked directly to the matrix prior to extraction and analysis, but for hair testing it is a little more complicated as the drug needs to be incorporated into the hair structure, rather than just coating the surface. Purchasing reference material for quality control and quality assurance is possible, but due to high demand and the difficulties in production, these samples can be hard to obtain and expensive [7]. Some laboratories prefer to source their own reference materials to be used, however these materials need to abide by the recommendations outlined by the Society of Hair Testing (SoHT) [15, 28, 29].

1.5 Conclusions and Project Premise

The analysis of drugs from hair samples can provide forensic toxicologists with a great deal of information that cannot be gained through the analysis of blood and urine. Hair analysis is usually performed as a complementary technique to blood and urine analysis as it has the added advantages of having a greater detection window (months to years, compared to hours to days). This longer detection window allows for determination of chronic drug use, chronic poisoning, the monitoring of drug abstinence and obtaining a drug history. Despite the added advantages, there are a number of issues associated with hair analysis, including the length of incubation (usually between 8-24 hours) required for sample preparation. This aspect is very time consuming, which can be a hindrance on the efficiency of the overall analysis process. Additional problems occur when the hair has been cosmetically treated, as this can interfere with either the analysis or the interpretation of results.

The aims of this research are:

1) To develop a fast and efficient method for the routine detection of drugs in hair, and to identify potential interferences with the hair analysis. Critical to this is the development of extraction methods with high recovery of drugs from the hair matrix. In particular, the use of microwave energy and ionic liquid composites were investigated for this purpose.

2) To determine the potential interferences in the routine detection of drugs in hair. Specifically, how the new restorative hair treatment, Olaplex®, can i) affect drug concentrations in hair, ii) interfere with the extraction process, and iii) how it can alter the overall structure of hair.

3) To develop hair reference standard material (drug incorporated hair) to assist with method development of hair analysis procedures and quality control.

Chapter 2

General Experimental

The reagents used during this project are stated in the list of Chemicals (pg. xxvi-xxviii). All the standard solutions and buffers were prepared in A-grade volumetric flasks. Solid samples were weighed on an AND GD-252 Electronic Balance (AND), and volumes of 500 µL or less were measured using an analytical glass volumetric syringe (SGE). All biological samples, i.e. hair, were handled, stored and disposed of according to the Forensic Science SA protocols and Southern Adelaide Clinical Human Research Ethics guideline.

2.1 Solvents and reagents

All solvents and reagents used were of analytical reagent grade or higher. Urea and choline chloride, used to make up the ionic liquid composite, were purchased from Sigma-Aldrich. Drug standards and internal standards were obtained from the National Institute of Forensic Science's Analytical Standards collection (New South Wales Forensic and Analytical Science Service Lidcombe, New South Wales, Australia) or Cerilliant Analytical Reference Standards (Novachem, Victoria, Australia). All drugs and drug metabolites used in this project were selected based on advice and recommendations of forensic practitioners at Forensic Science SA (FSSA). Precision glass syringes (SGE, Australia) and a calibrated analytical pan balance were used where appropriate.

2.1.1 Calibration solution and internal standard concentrations

To calculate the concentration of drugs in hair, a calibration curve was produced using a calibration mix. Specific volumes were spiked to hair samples and extracted using the 18-hour incubation method in methanol (2 mL at 45 °C, refer to section 2.3). The concentrations for each analyte and the different calibration levels are listed in Table 3. Cal 1 is a blank (un-spiked) sample. A calibration curve (with a 1/x weighting) is produced using the known concentration of the area ratio (analyte/IS), and the concentration of the unknown determined also based on the area ratio (analyte/IS). The internal standard (IS) concentrations are also present in Table 3. This is the normalised calculated concentration based on the assumption 20 mg of the hair sample was used. Actual concentration of each drug is based on the actual mass of the hair sample which is calculated using Equation 1.

Equation 1: Calculation of concentration of drug in hair using the actual mass of hair sample

$$Concentration\ (pg/mg) = \frac{\text{calculated}\ concentration\ (pg/mg)\ x\ 20\text{mg}}{actual\ mass\ (mg)}$$

Table 3: Calibration concentrations (pg/mg) at the varying calibration levels and internal standard (IS) concentrations (pg/mg) when spiked (20 µL) to the organic extract prior to evaporation and reconstitution

Target Analytes	**Cal 2**	**Cal 3**	**Cal 4**	**Cal 5**	**Cal 6**
Amphetamine	12.50	31.30	125.0	312.5	625.0
MDMA	12.50	31.30	125.0	312.5	625.0
Methamphetamine	12.50	31.30	125.0	312.5	625.0
Morphine	12.30	30.60	122.5	306.3	612.5
Codeine	11.90	29.70	118.8	296.9	593.8
Temazepam	26.10	65.10	260.6	651.4	1303
Oxazepam	25.60	63.90	255.5	638.8	1278
Nordiazepam	23.40	58.40	233.5	583.8	1168
Diazepam	25.00	62.50	250.0	625.0	1250
Methadone	11.40	28.60	114.4	286.0	572.0
Cocaine	12.50	31.30	125.0	312.5	625.0
Oxycodone	10.00	25.00	100.0	250.0	500.0
Internal Standards	**IS**				
D3 Morphine	125				
D5 MDMA	100				
D4 Pethidine	125				
D3 Codeine	119				
D5 Methamphetamine	150				
D5 Amphetamine	125				
D3 Cocaine	125				
D5 Diazepam	1650				
D5 Oxazepam	450				

2.2 Hair sample collection and preparation

The use and collection of human hair samples for this project was approved by Southern Adelaide Clinical Human Research Ethics Committee (application 166.14), on June 6, 2014. Hair samples collected for method development sections were collected as hair trimmings (i.e. from the tip of the hair shaft), or locks cut from the scalp from healthy volunteers and screened for drugs prior to use. Due to restriction with ethics, the collection of the hair samples had to be conducted by the volunteer themselves, placed in a zip lock bag, and deposited, anonymously, in a collection box. Hence for samples collected as locks of hair from the scalp, the proximal distance of collection was not

recorded. The only way to include proximal distance for analysis is for volunteers to pluck their own hair, which would be painful for collection of a large number of hairs and invasive for the volunteers. Therefore, that approach was not considered. Samples were stored in either aluminium foil, or in separate zip lock bags, in a cupboard at room temperature, away from light. The hair samples collected and used over the duration of the project are listed in Table 4. To remain consistent, unless otherwise specified, each sample used approximately 20 mg of hair.

Table 4: Hair samples collected from volunteers, including condition of hair at time of donation, cosmetic treatments applied to the hair within the last 12-month and any drugs administered to the volunteer within the last 12 months

Sample	Reference number	Sample type	Treatments	Drugs disclosed
1	H14-02	Pencil thick locks of hair	Bleached	N/A
2	H14-18	Trimmings	Blonde (dyed). Blow dried every 2nd day	N/A
3	H14-09	Trimmings	Blonde, bleached and coloured. Blow dried every morning	N/A
4	H14-15	Full ponytail cut off	Light brown/blonde. No chemical or heat treatments applied	N/A
5	H15-20	Trimmings	Dark brown. Straightened once a month	N/A
6	H14-01	Shaved	Dark brown. No chemical or heat treatments applied	N/A
7	H14-06	Trimmings	Brown (coloured), Straightened ~3 times a month	Paracetamol, ibuprofen
8	H14-19	Pencil thick locks	Dark brown. No chemical or heat treatments applied	Paracetamol, Ibuprofen, codeine
9	H14-07	Trimmings	Dark brown. Blow dried 3 time a week	N/A
10	H17-15	Full ponytail cut off	Light brown/blonde. No chemical or heat treatments applied	N/A
11	H17-22	Trimmings	Dark brown, coloured frequently, blow dried 3 times a week	Oxazepam and temazepam (taken weekly). Diazepam taken on occasion
12	Sample A	Proficiency sample (pre-determined concentrations)	Brown hair. Potentially from multiple donors. No other information provided	Amphetamine (~200 pg/mg) Methamphetamine (~400 pg/mg) MDMA (~600 pg/mg) Morphine (~300 pg/mg) Codeine (~70 pg/mg) Diazepam (~15 pg/mg) Methadone (~50 pg/mg) Cocaine (~5000 pg/mg)

To prepare spiked hair samples, a standard drug mix (DMIX HAIR) containing morphine (MOR), codeine (COD), amphetamine (AMP), methamphetamine (METH), MDMA, methadone (MET), cocaine (COC), oxycodone (OXY), diazepam (DIAZ), nordiazepam (NOR), oxazepam (OXAZ) and temazepam (TEM) was prepared by dissolution in methanol/dimethyl sulphoxide (DMSO) (1:1, v/v) at 1 µg/mL for all drugs (refer to List of Chemicals for drug concentrations in DMIX). Drug free hair samples collected from 3 donors (samples 2, 5 and 9, 1 g each, finely cut into 1-5 mm fragments) were washed with methanol (20 mL) and spiked with analyte based on a method described by Martin *et al.* [133]. The cut hair was soaked in the DMIX HAIR solution (20 mL) for 7 days under nitrogen atmosphere and away from light. The DMIX HAIR was then removed, and the spiked hair was washed a further ten times. The final wash was collected and analysed to ensure any remaining analyte was incorporated into the hair, rather than coating the outer cuticle layer.

2.3 Extraction and clean-up methods

Unless otherwise stated all hair samples were prepared for analysis using the following overnight incubation method which is currently used at Forensic Science SA for hair analysis. Hair (20 mg, finely cut into 1-5 mm fragments) was swirled in methanol (2 mL) for 30 seconds to remove contaminants. This methanol wash can be collected and analysed to ensure the hair sample was contaminant free. Methanol (2 mL) is then added to the clean hair and incubated at 45 °C for 18 hours, in a Contherm Scientific Ltd. Designer Series oven. The samples were then left to cool to room temperature, vortexed, and methanol collected and transferred into new, clean glass tube. Followed by the addition of acid alcohol (20 µL; 50 µL HCl: 10 mL MeOH) and internal standard (20 µL). The solvent is evaporated off under N_2 steam at 35 °C, and reconstituted in 100 µL of 0.1 % formic acid (prepared with Optima LC grade formic acid in Milli-Q water). Reconstituted extract is transferred into a LC vial, fitted with a 250 µL glass insert.

2.4 Instrumentation

2.4.1 Microwave reactor

Microwave digestion and extraction (for all applicable sections) was performed with a CEM Discover SP microwave (closed, single vessel). The pressurized microwave oven uses 10 ml Pyrex® vessels, with simultaneous magnetic stirring. Specific microwave conditions are described in relevant chapters.

2.4.2 Q TRAP 4000

The majority of the analysis and drug detection was performed on a SCIEX 4000 QTRAP® LC-MS/MS system. This system uses a hybrid triple quadrupole/linear ion trap mass spectrometer. Chromatographic separation and mass spectrometer details are provided in Table 5, and monitored transition ion details for each analyte are provided in Table 6.

Table 5: Separation and detection conditions for the SCIEX QTRAP® LC/triple-quad system

LC system	
Analytical column	Phenomenex Luna® PFP(2) LC column (3µm 50 x 4.6mm)
Guard column	Phenomenex 4x2mm PFP Security Guard™ cartridge
Mobile phase A	Methanol
Mobile phase B	0.1% Formic Acid (in Milli-Q water) – remade daily
Initial 0.5 min Gradient 0.5-6.5 min Isocratic 6.5-10.1 mins Gradient 10.1-10.5 mins Final 10.5-14 min	95 % B 95 % B→0 % B 0 % B 0 % B→95 % B 95 % B
Flow rate	0.75 mL/min
Column temp	25 °C
Injection volume	5 µL (unless otherwise stated)
Run time	14 min
QQQ	
Scan type	Multiple reaction monitoring (MRM)
Ion Source	Turbo Spray
Polarity	Positive
Data acquisition and analysis software	
Data acquisition	Analyst
Data analysis	MulitQuant 2.1

Table 6: List of target analytes and the retention times and monitored Ions for LC-QTRAP® 4000 analysis

ID	Retention time	Q1	Q3
Amphetamine 1	5.2	136.0	119.0
Amphetamine 2	5.2	136.0	91.0
Amphetamine 3	5.2	136.0	65.0
MDMA 1	5.8	194	163
MDMA 2	5.8	194	105
MDMA 3	5.8	194	133
Methamphetamine 1	5.4	150	119
Methamphetamine 2	5.4	150	91
Methamphetamine 3	5.4	150	65
Morphine 1	3.3	286	201
Morphine 2	3.3	286	229
Morphine 3	3.3	286	153
Codeine 1	4.4	300	215
Codeine 2	4.4	300	225
Codeine 3	4.4	300	165
Temazepam 1	7.0	301	255
Temazepam 2	7.0	301	177.1
Temazepam 3	7.0	301	283
Oxazepam 1	6.7	287	104
Oxazepam 2	6.7	287	163
Oxazepam 3	6.7	287	296
Nordiazepam 1	7.0	271	208
Nordiazepam 2	7.0	271	140
Nordiazepam 3	7.0	271	165
Diazepam 1	7.1	285	193
Diazepam 2	7.1	285	222
Diazepam 3	7.1	285	257
Methadone 1	7.2	310	265
Methadone 2	7.2	310	105
Methadone 3	7.2	310	76.8
Cocaine 1	6.9	304	182
Cocaine 2	6.9	304	105
Cocaine 3	6.9	304	82
Oxycodone 1	4.9	316	298
Oxycodone 2	4.9	316	212
Oxycodone 3	4.9	316	241.5
D5-Diazepam	7.1	290	198
D3-MAM	4.9	331	211
D3-Morphine	3.3	289	201
D3-Codeine	4.4	303	215
D5-MDMA	5.8	199	165
D5-methamphetamine	5.4	155	121
D5-Amphetamine	5.2	141	93
D4-Pethidine	6.8	252	224
D3-Cocaine	6.9	307	185
D5-Oxazepam	6.7	292	246

2.4.3 LC-QTOF

Analysis and drug detection (where specified) was performed on an Agilent Technologies 1200 LC system coupled to a 6520 quadrupole-time-of-flight mass spectrometer (QTOF). The LC-QTOF was used instead of the LC-QQQ for Chapter 4 as it is capable of performing a general screen, rather than a targeted analysis. The columns available for the LC-QTOF were also more suitable for ionic liquid composite analysis. Separation and detection details are provided in Table 7, and details of retention times and monitored ions are provided in Table 8.

Table 7: Separation and detection conditions for the Agilent Technologies 1200 LC system coupled to a 6520 quadrupole-time-of-flight mass spectrometer (QTOF)

LC system	
Analytical column	Waters Aquity UPLC® BEH C18 1.7 µm 3.0x50 mm *BEH= ethylene bridged hybrid
Guard column	Phenomenex 3x4mm C18 Security Guard™ cartridge
Mobile phase A	Acetonitrile
Mobile phase B	0.1% Formic Acid (in Milli-Q water) – remade daily
Initial 0.5 min Gradient 0.5-7.5 min Gradient 7.5-10 mins Final 10-12 min	90 % B 90 % B→50 % B 50 % B→5 % B 0 % B
Flow rate	0.35 $mLmin^{-1}$
Column temp	30°C
Injection volume	10 µL (unless otherwise stated)
Run time	12 min
Post run	2.5 min
QTOF	
Instrument State	Low mass range, extended dynamic range
Ionisation mode	ESI
Polarity	Positive
Collision gas	Nitrogen (350 °C, 10 L/min)
Nebulizer pressure	50 psi
Capillary voltage	3.0 kV
Fragmentor	125 V
MS mode	TOF only acquisition mode
Data acquisition and analysis software	
Agilent Mass Hunter Qualitative and Quantitative Analysis version 4.0.	

Table 8: List of target analytes and the retention times and the mass of the monitored m/z for LC-QToF analysis

Drug	RT	Monitored ion (±20 mDa)
Amphetamine	3.03	136.1121
MDMA	3.71	194.1176
Methamphetamine	3.51	150.1277
Morphine	1.18	285.3411
Codeine	2.36	300.1594
Temazepam	9.28	301.7079
Oxazepam	8.37	287.0582
Nordiazepam	8.64	271.0633
Diazepam	9.87	285.0789
Methadone	8.35	310.2166
Cocaine	5.62	304.1543
Oxycodone	3.21	316.1544

2.4.4 Microscopy

Light-Transmitted microscopy

Light-Transmitted microscopy was performed using an Olympus System CX41 Compound microscope (at 100x magnification). Microscope was equipped with an Olympus DP21 2 megapixel digital camera for imaging.

SEM

Scanning electron microscopy (SEM) was performed using a Inspect FEI F50 SEM. Unless otherwise specified each analysis used 10 kV ion beam and a spot size of 5, with the sample set 11 mm from the ion beam. Prior to SEM analysis, dried hair fibres were mounted on carbon tape and sputter coated with 10 nm gold, using a Q300T-D Dual Target Sputter Coater.

FT-IR and ATR microscopy

Fourier-transform infrared (FT-IR) microscopy was performed using Nicolet™ Nexus 870 FT-IR, coupled with Nicolet™ Continuum™ FT-IR microscope. The microscope was also equipped with a liquid nitrogen cooled, narrow band MCT detector. Prior to analysis the hair fibre is flattened (to < 10 micron think) between two diamond plates using Thermo Scientific's Micro Compression Cell II with diamond windows. Analysis is conducted with flattened hair mounted on one of the diamond windows. ATR was also performed on the

Nicolet™ Nexus 870 FT-IR, coupled with Nicolet™ Continuum™ FT-IR microscope using a Germanium ATR crystal. A hair fibre was mounted on the adhesive strip of a 3M post-it note, which was secured to a stainless-steel microscope slide with a piece of double-sided sticky tape (Sellotape).

2.4.5 ToF-SIMS

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) analysis was performed using a Physical Electronics, Inc. PHI TRIFT V nanoToF, equipped with a pulsed liquid metal 79+Au primary ion gun (LMIG), in positive mode, operating at 30 keV energy. All experiments were performed under a vacuum of $5x10^{-6}$ Pa.

2.4.6 ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) analysis was performed on a PerkinElmer NexION 350D ICP-MS. The details and conditions of the ICP-MS are present in Table 9. The analysis was performed in KED (Kinetic Energy Discrimination) mode with Internal Standard correction using Indium. The internal standard was added externally through a T-junction for standard and samples. Elemental standards were supplied by High Purity Standards at 1000ppm concentration and diluted to appropriate ranges.

Table 9: Instrumental details for ICP-MS analysis using Perkin Elmer NEXION 350D ICP-MS

ICP-MS System	
Spray chamber	quartz cyclonic temperature controlled at 2 °C
Nebuliser	glass concentric 300 µl/min
RF power	1600 W
Plasma flow	16 L/min
Auxiliary flow	1.2 L/min
Nebuliser flow	0.9 L/min
Dwell time	100 ms with 3 replicates
Helium flow	4.7 mL/min
Injection rates	
Sample	0.25 mL/min
Internal standard	0.02 mL/min

2.5 Glassware

All the standard solutions and buffers were prepared in A-grade volumetric flasks. In preparation for IC-PMS analysis, glassware was soaked overnight in 5 % nitric acid solution (trace grade nitric acid) made up in ultra-pure Milli-Q water. This process was repeated 3 times with fresh 5 % nitric acid solution, and an ultra-pure Milli-Q water rinse in between soaks. After third overnight soak glassware was rinsed and incubated for 1 hour at 100 °C for drying. In preparation for all other experiments, glassware was washed thoroughly with ethanol, and when possible, dried in the oven at for at least 1 hour at 100 °C.

Chapter 3

Microwave-Assisted Extraction

This chapter investigates the use of microwave assisted extraction (MAE) to replace the traditional overnight (usually 8-24 hours) incubation. The results discussed in this chapter include:

- *Method development including the examination of the effect of microwave time and temperature*
- *Method validation, including drug recovery, matrix effects and comparison to 18-hour incubation at 45°C*
- *Investigations into the stability of target analytes throughout microwaving process, and in the final extract*
- *Assessment of the potential for contamination and carry-over through re-using microwave extraction tubes*

3.1 Introduction

Despite the advantages of hair testing, there are a number of drawbacks associated with it, one of which is the length and complexity of the sample preparation. Whilst there are currently no internationally recognised standard methods for hair analysis, the Society of Hair testing (SoHT) have published guidelines and recommendations to assist laboratories with their methodologies and interpretations [15, 28, 31, 32, 134, 135]. The general procedure consists of decontamination, extraction, clean-up and instrumental analysis steps, with each step varying significantly between laboratories. Extraction procedures can vary in the type of solvent, the volume, temperature of incubation and the length of incubation. For example, methanol extraction has been reported to take 5-18 hours in a sonication bath [68]. The overall extraction aspect of the procedure is very time consuming, which can be a hindrance on the efficiency of the overall analysis process.

Microwave energy has recently been used as an alternative to overnight/lengthy incubations to reduce the time required for the extraction. Microwave energy has been widely used in analytical laboratories for its ability to rapidly heat materials. Abu-Samra *et al.* (1975) were the first to use microwave energy in an analytical laboratory, and this was for the trace analysis of metals from biological samples [136], with numerous applications of microwave assisted extraction (MAE) published since this report. MAE involves the analyte(s) of interest being transferred from the sample matrix to an organic solvent with the assistance of microwave irradiation. The microwave irradiation causes the sample and solvent to be heated via two mechanisms: ionic conduction and/or dipole rotation [137]. Ionic conduction refers to electrophoretic migration of ions, whereas dipole rotation is the realignment and randomisation of dipoles. These forced molecular movements cause the sample to heat. With commercial microwave systems (at 2450 MHz), the dipoles can align and randomise up to 4.9×10^9 times per minute [138].

MAE has frequently been used for the extraction of organic pollutants from sediments, soil and water [139], and even the extraction of drugs from tablets [140]. Recent studies have indicated potential use for MAE in the field of clinical and forensic toxicology, which enables the length of time required for the digestion/extraction procedure to be reduced from hours to minutes. MAE has been used in a number of studies to detect drugs, such as ethyl glucuronide [141, 142], ketamine and its metabolites [143], cocaine and opiates

[67, 144-147], tricyclic antidepressants [148-150], and benzodiazepines [137, 151, 152] in various biological matrices, including hair (refer to Table 10). It should be noted that the stimulant drug class has currently not been assessed for MAE (apart from cocaine and its metabolites).

Table 10: Drug classes that have successfully been extracted from biological materials using MAE and the optimised conditions at which they were extracted

Drug class	Biological matrix	Solvent	Extraction temp (°C)	Extraction time (min)	Reference
Anaesthetic (including: ketamine & norketamine)	Bone (rat)	Acetone-hexane (1:1 v/v)	80	30	[143]
CNS depressants (including: ethyl glucuronide)	**Hair**	***n*-Hexane/water (1:1 *v/v*)**	**110**	**11**	**[141]**
	Urine, 0.5mL	Chloroform; 5mL	90	3	[142]
analgesic and anti-inflammatories (including: Salicylic acid, acetaminophen, metamizol, tramadol, Ibuprofen & diclofenac	Urine	Ethyl acetate; 12 mL (with pH 4.0 phosphate buffer; 4 mL)	115	4	[153]
	Plasma	Ethyl acetate; 12 mL (with pH 4.0 phosphate buffer; 4 mL)	67	9	[153]
Opioids and non-analgesics * (including: Morphine, 6-acetylmorphine, codeine, cocaine, cocaethylene, benzoylecgonine, methadone, EDDP & 6-MAM	**Hair**	**Dichloromethane (MeOH as a modifier)**	**60**	**9**	**[67]**
	Urine; 1mL	Chloroform; 10 mL (pH modified up to 9.0)	100	10	[144]
	Plasma	Chloroform	75	10	[145]
	Saliva; 1mL	Chloroform; 10 mL	100	10	[146]
	Vitreous humour; 0.5 mL	Dichloromethane; 15 mL	80	8	[147]
Benzodiazepines* (including: alprazolam, bromazepa, lorazepam, lormetazepam, tetrazepam, estazolam, clonazepam & diazepam	**Hair**	**Ethyl acetate; 3 mL (pH 9.5 borate buffer; 980 µL)**	**75**	**10**	**[137]**
	Plasma; 1mL	Chloroform/2-propanol (4:1 v/v); 8 mL (with pH 9.0 borate buffer; 2 mL)	89	13	[151]
	Vitreous humour; 0.5 mL	Ethyl acetate; 10 mL (with pH 9.0 borate buffer; 2 mL)	98	10	[135]
Tricyclic antidepressants (including: TCAD, nordoxepin, nortriptyline, imipramine, amitriptyline, doxepin, desipramine, clomipramine & norclomipramine	Serum	*n*-Hexane-isoamyl alcohol (99:1 v/v)	60	1	[148]
	Hair	***n*-hexane/isoamyl alcohol (99:1, *v/v*)**	**60**	**40**	**[149]**

*Analytes previously tested that are being investigated in this study include morphine, codeine, cocaine, methadone and diazepam. It should be noted that no stimulants (apart from cocaine and its metabolites) had been investigated in the use of microwave-assisted extraction, at the time of this project.

Additionally, Wietcha-Posluszny *et al.* (2011) has demonstrated a simultaneous alkaline microwave-assisted hydrolysis with microwave-assisted extraction (MAH-MAE) from hair [149]. These studies have all reported the benefits of using microwave energy to assist extraction of drugs from biological materials are a significant reduction in solvent consumption and extraction time compared to traditional methods, without compromising recovery. Another major benefit of MAE for forensic and clinical analysis is the potential for it to be a one-step procedure. As MAE usually involves extraction into an organic solvent, no additional clean-up step is needed. The organic solvent is simply collected, evaporated to dryness and reconstituted. MAE has even been referred to as clean and green technology due to the reduced waste produced, compared to other methods [151].

Currently the extraction method used at Forensic Science SA (the collaborating partners in the project) is an 18-hour incubation at 45 °C, making it very time consuming. The long extraction time can greatly affect the turnover rate for cases, resulting in back log. Already there is a huge demand and slow turnaround time for hair analysis at Forensic Science SA. In 2014 approximately 40 case segments were analysed per month, with each case having a 30-day turnaround (unpublished data). If the 18-hour incubation time could be reduced this would greatly increase case reporting times and reduce back log. In this chapter MAE was implemented to improve upon the method currently used at Forensic Science SA, substituting the usual 18-hour digestion at 45 °C for microwave heating in an attempt to reduce extraction time.

3.2 Experimental

3.2.1 General

Microwave-assisted extraction was performed with a CEM Discover SP microwave (closed, single vessel) and all extracts were analysed using the SCIEX QTRAP® 4000 LC-MS/MS system (refer to Chapter 2.4 for details).

3.2.2 Hair sample collection and preparation

Hair samples from three different donors (Samples 1, 4 and 8, refer to Table 4, Chapter 2.2) were collected and screened prior to use to ensure all samples were drug-free. Each hair sample (1 g, finely cut into 1-5 mm fragments) was washed with methanol (20 mL) and spiked with analyte based on a method described by Martin *et al.* [133] (refer to Chapter 2.2). Briefly, the cut hair was soaked in the standard drug mix solution prepared by dilution in methanol/dimethyl sulphoxide (DMSO) (20 mL, 1:1 v/v) at 1 µg/mL for 7 days under nitrogen atmosphere and away from light. The drug mix was then removed, and the artificially incorporated hair was washed a further ten times with methanol to ensure any remaining analyte was incorporated into the hair and not coating the surface of the hair.

3.2.3 Extraction procedure and method development

For each sample, 20 mg of each donor's artificially drug incorporated hair were weighed out in separate, clean extraction tubes, and submerged in methanol (2 mL) and extracted (either through MAE or 18-hour oven incubation at 45 °C). Following extraction, the methanol was collected for evaporation to dryness under a N_2 stream at 40 °C. The IS was added to the methanol immediately prior to evaporation. The dry extract was then reconstituted in 0.1 % v/v aqueous formic acid (100 µL) and analysed. The current hair analysis method used at Forensic Science SA is 18-hour incubation, 20 mg hair in 2 mL methanol, at 45 °C. The extraction solvent for MAE was chosen as methanol (2 mL) to stay consistent with the current extraction solvent used. Hence, only extraction time and temperature were assessed for the microwave conditions. Microwave temperatures assessed ranged between 55 °C - 75 °C, at 5 °C intervals, and microwaving time between 1 and 180 minutes at various intervals.

3.2.4 Comparison to current overnight method and validation

The MAE method using 2 hours at 70 °C was compared to the current overnight incubation method. This was done using the 3 artificially incorporated hair samples, as well as an additional artificially incorporated hair sample (used in Chapter 5), each in triplicate. A comparison was also done using authentic hair samples containing one or more of the analytes of interest (samples 11 and 12, refer to Table 4, Chapter 2.2).

A validation study was carried out which investigated linearity, recovery, matrix effects, and inter-day precision, accuracy, bias and selectivity. Intra-day precision was not conducted due to limitations with the capacity of the microwave, which was restricted to single samples. Linearity was assessed by extracting and analysing 20 mg hair spiked at all 5 concentrations listed in Table 11, in triplicate, using the MAE method to produce a calibration curve based on the peak area ratio (analyte/IS) and analyte concentration. The recovery, matrix effects, precision, accuracy and bias of each analyte was investigated using 20 mg of drug-free hair spiked at 3 concentrations (concentrations 1, 2 and 3 listed in Table 11) in triplicate. Standard drug solutions were added to the drug-free hair prior to microwaving, and the internal standard was added immediately before evaporation to dryness after microwaving. Each assessment was calculated according to Equation 2, 3, 4, and 5 below.

The analysis for the matrix effects was carried out using an auto-sampler co-injection method described by Rogers *et al.* [154]. Briefly, instead of conducting a manual *post-extraction spike*, where a known concentration drug standard is added to the extract prior to analysis, the drug standard was added to the blank extract using an automated co-injection method on the LC-QTrap, whereby the injection needle drew up a set volume of the drug-free hair extract, followed by a set amount of the known concentration drug standard (in a separate vial in the auto-sampler). The extract and drug standard were then mixed inside the needled immediately prior to injection.

The hair samples used for all validation experiments were from the same three hair donors (hair samples 1, 4 and 8) used previously in the method development stage. The possibility of false-positive results was assessed through the analysis of 10 blank hair samples collected from volunteers (samples 1-10, refer to Chapter 2.2), using MAE for 2

hours at 45 °C. All hair samples had been confirmed to be blank prior to this assessment, except sample 8 which contained codeine. This was also declared by the donor prior to submitting the sample.

The potential for contamination through re-using the microwave extraction tubes was assessed by microwaving blank methanol (2 mL) in 5 different extraction tubes which had all been used for previous samples, and calculating the concentration of each analyte as per the methods discussed above. The tubes had all been washed according to the wash procedures described in Chapter 2.3. This procedure of microwaving blank methanol (2 mL) was then repeated using new, unused microwave extraction tubes to rule out other sources of contamination.

Table 11: The concentrations (pg/mg) used to spike samples for the validation and stability studies when spiked with 10 µL, 25 µL, 250 µL, 500 µL and 1000 µL of the drug mix

	Concentration 1 (10 µL spike)	Concentration 2 (25 µL spike)	Concentration 3 (250 µL spike)	Concentration 4 (500 µL spike)	Concentration 5 (1000 µL spike)
AMP	12.5	31.3	313	625	1250
MDMA	12.5	31.3	313	625	1250
METH	12.5	31.3	313	625	1250
MOR	12.3	30.6	306	613	1225
COD	11.9	29.7	297	594	1188
TEM	26.1	65.1	651	1303	2606
OXAZ	25.6	63.9	639	1278	2555
NOR	23.4	58.4	584	1168	2335
DIAZ	25.0	62.5	625	1250	2500
MET	11.4	28.6	286	572	1144
COC	12.5	31.3	313	625	1250
OXY	10.0	25.0	250	500	1000

Equation 2: Percentage recovery of target analytes

$$Recovery = \left(\frac{Spike\ area\ ratio}{Neat\ standard\ area\ ratio}\right) * 100$$

Equation 3: Calculation of potential for matrix effects (ion suppression or enhancement)

$$Matrix\ Effects = \left(\frac{Co - injection\ area\ ratio}{Neat\ standard\ area\ ratio}\right) * 100$$

Equation 4: Calculation of precision for method validation

$$Precision = \left(\frac{standard\ deviation\ (C_{determined})}{mean\ (C_{determined})}\right) * 100$$

Equation 5: Calculation of bias for method validation

$$Bias = \left(\frac{C_{expected} - C_{determined}}{C_{expected}}\right) * 100$$

Spike area ratio = peak area ratio (analyte/IS) in the sample spiked prior to microwaving

Neat standard area ratio = peak area ratio (analyte/IS) of the neat standard

Co-injection area ratio = peak area ratio (analyte/IS) of the extracted sample co-injected with neat drug mix

$C_{determined}$ = mean value of calculated concentration (pg/mg)

$C_{expected}$ = known concentration spiked to sample (pg/mg)

3.2.5 Assessment of analyte stability

Stability of the analytes in the microwave was assessed through the comparison of a neat standard that had been microwaved, with a neat standard that had been kept at room temperature. Blank methanol (2 mL) was spiked with the drug mix concentrations 1-3 presented in Table 11, and microwaved at the optimised conditions (70 °C for 2 hours), in triplicate. The stability of each analyte was calculated according Equation 6 below. The stability of the analytes in the extract was also assessed by analysing the MAE samples of each of the blank hairs spiked at the 3 concentrations one day and one week after the original analysis. The same was done for the neat standards at the same concentrations, in triplicate. The stability of each analyte in the extract was calculated according Equation 7 below. The stability of each analyte in the extract was also calculated according Equation 8 below. The analyte/IS ratios were not used in this instance, in case the internal standard was also degrading over the storage period. The results for this method of determining extract stability are presented in the Appendix.

Equation 6: Calculation for the stability of target analytes in the microwave during extraction

$$Stability = 100 - \left(\frac{Spike\ area\ ratio}{Post\ microwave\ spike\ area\ ratio} * 100\right)$$

Equation 7: Calculation for the stability of target analytes in the extract after 1 day or 1 week in storage, using the analyte/IS ratio

$$Extract\ Stability = 100 - \left(\frac{Spike\ area\ ratio\ (after\ 1\ day\ or\ 1\ week)}{Spike\ area\ ratio\ (initial)} * 100\right)$$

Equation 8: Calculation for the stability of target analytes in the extract after 1 day or 1 week in storage, using the analyte area

$$Stability = 100 - \left(\frac{Spike\ area\ (after\ 1\ day\ or\ 1\ week)}{Spike\ area\ (initial)} * 100\right)$$

Spike area ratio = peak area ratio (analyte/IS) in the sample spiked prior to microwaving

Post microwave spike area ratio = peak area ratio (analyte/IS) in the sample spiked after microwaving

Spike area = area of target analyte

3.3 Results and Discussion

3.3.1 Assessment of microwave conditions

Initial experiments conducted assessed the variation in extracted concentration with increased temperature, with extraction being carried out between 55 °C and 75 °C, at 5 °C intervals (refer to Table 12 for detected concentrations). Methanol has a boiling point of 64.7 °C, however since the microwave is a pressurized vessel, the temperature of the sample is able to be pushed beyond the normal atmospheric pressure boiling point.

It is important to note that as the amount of drug that is incorporated into the hair during sample preparation is not known, it is not possible to indicate what the expected maximum extractable concentration is. However, each hair sample was treated with the same concentration of drug (20, 000 pg/mg of hair), so for the initial method development stage, extracted concentrations of each drug for each time and temperature point were compared relative to each other. This was to determine which conditions extract the most amount of drug from the hair. A higher concentration of drug extracted indicates a better extraction method. Later in the study, the chosen microwave method is compared to the current extraction method to determine if the method meets minimum requirements (on page 55). Again, the extracted concentrations were compared relative to each other, rather than to an expected maximum concentration.

As shown in Table 12, there is a noticeable increase in detected concentration for all of the drugs assessed with the increase in microwave temperature; hence more of the analyte is being extracted with the increase in temperature. For some of the analytes however, there was a decrease in the extracted concentration between the 70 °C and 75 °C microwave temperatures. For hair 1 and 4 samples, the concentrations of methadone, nordiazepam and diazepam were lower in the 75 °C extractions, compared to the 70 °C extractions. Whereas in the hair 8 sample, MDMA, morphine, codeine, temazepam, nordiazepam, diazepam and methadone all had lower concentrations in the 75 °C extractions, compared to the 70 °C extractions. Benzodiazepines, such as temazepam, diazepam and nordiazepam, are known to be thermally unstable, and as such, it is not unexpected that these analytes would undergo thermal degradation at these high temperatures. However, it is unclear from this experiment whether these drugs are in fact undergoing degradation, or are simple not being extracted as well.

Additionally, there are a number of drugs including diazepam (in hair 1), MDMA and cocaine (in hair 4), oxycodone (in hair 4 and 8), and oxazepam (in hair 8), that have minimal or no increase in the detected concentrations in the 75 °C extractions, compared to the 70 °C extractions.

Following this, extraction at various time intervals between 1 and 180 minutes at 70 °C was assessed. 70 °C was chosen as the set temperature for this step to allow for as much extraction of the analyte as possible while minimizing the potential degradation that could occur at higher temperatures. As seen with the increase in temperature, the increase in extraction time resulted in higher detected concentrations for all drugs assessed (refer to Table 13). For the hair with the least amount of incorporated drug (hair 8), amphetamine was detected but at levels below the limit quantification, and was not detected after 1 minute, and was only detected above the limit of quantification after 45 minutes. For three of the drugs, amphetamine, nordiazepam and diazepam, the maximum detected concentration was reached after 60 minutes, but the remaining drugs 9 drugs required at least 2 hours of microwaving to reach the maximum extracted concentration. For the other two hair samples with the higher concentration of analyte (hair 1 and hair 4), every analyte was detected above the limit of quantification after only 1 minute. For hair 1, temazepam, oxazepam, nordiazepam, diazepam, methadone, cocaine and oxycodone all reached the maximum concentration between 30 minutes to 1 hour, while amphetamine, MDMA, methamphetamine, morphine and codeine all required 3 hours to reach the maximum. For hair 4, only nordiazepam, diazepam and methadone reach maximum concentration within the first hour, with diazepam reaching maximum after only 10 minutes. The remaining drugs again required 3 hours. By 4.5 hours the concentration had decreased for all analytes. This may be due to the drug degrading.

Two hours was chosen as the preferred time to use as it allowed for enough time to extract a sufficient amount of the target analyte. It was decided that increasing the time further did not results in enough of an increase in the extracted concentration to the be considered beneficial. Additional experimental parameters that could be assessed in the future to further improve the extraction procedure include ball-milling or pulverization of the hair instead of cutting to increase the surface area to volume ratio and alternate extraction solvents.

Table 12: Effects of temperature on calculated concentration (pg/mg), using microwave-assisted extraction (MAE) to extracted drugs from artificially incorporated hair samples. Prior to extraction, each hair sample was spiked with a drug solution that contained 20,000 pg of drug per mg of hair.

	Temp	AMP	MDMA	METH	MOR	COD	TEM	OXAZ	NOR	DIAZ	MET	COC	OXY
Hair 1	55 °C	263	489	242	54.7	57.5	142	48.4	117	122	71.9	32.0	65.1
	60 °C	296	539	287	68.1	72.6	172	54.2	138	150	106	42.1	80.5
	65 °C	270	609	286	79.6	79.7	179	57.8	145	153	125	48.4	91.6
	70 °C	318	616	284	96.7	93.7	192	62.7	136	151	131	49.5	106
	75 °C	363	710	301	104	109	204	66.8	173	156	121	55.9	113
Hair 4	55 °C	96.9	139	116	33.8	34.6	106	29.0	94.3	128	29.4	19.1	29.4
	60 °C	122	174	123	49.5	58.4	146	41.2	132	161	37.8	27.9	45.7
	65 °C	109	184	121	62.7	66.8	137	41.0	127	166	36.5	30.7	53.0
	70 °C	139	228	124	73.0	79.6	165	47.7	135	177	46.6	34.1	67.1
	75 °C	130	230	137	79.7	85.3	173	54.2	125	170	53.2	34.1	69.8
Hair 8	55 °C	52.6	14.2	59.5	26.5	28.9	99.7	32.8	99.7	156	17.7	<LOQ	39.2
	60 °C	13.8	13.5	48.8	22.9	26.0	98.7	29.5	95.1	148	17.9	<LOQ	31.9
	65 °C	15.2	14.3	54.7	32.6	30.3	112	34.3	100	161	23.6	<LOQ	38.5
	70 °C	18.3	20.6	49.9	38.6	44.1	100	28.7	111	169	21.8	<LOQ	41.8
	75 °C	23.9	17.9	63.2	34.6	37.3	96.0	28.7	95.7	131	17.7	<LOQ	41.2

*NB: For drugs that were detected, but below the LOQ, results reported as <LOQ. For drugs that were not detected at all, the cell will be greyed out.

Table 13: Effect of extraction time on calculated concentration of analyte (pg/mg) using microwave-assisted extraction (MAE) to extracted drugs from artificially incorporated hair samples. Prior to extraction, each hair sample was spiked with a drug solution that contained 20,000 pg of drug per mg of hair.

	Time (min)	AMP	MDMA	METH	MOR	COD	TEM	OXAZ	NOR	DIAZ	MET	COC	OXY
Hair 1	1	332	665	288	86.2	75.5	93.0	68.6	161	178	102	47.8	93.9
	10	408	752	299	127	112	109	86.7	169	187	136	61.6	147
	20	438	878	370	163	140	118	94.8	182	191	159	65.1	160
	30	528	1040	401	179	164	131	107	203	206	155	73.1	203
	45	633	1250	451	215	183	145	126	122	125	238	70.2	266
	60	613	1190	447	220	178	129	117	193	191	178	75.6	233
	120	611	1260	456	242	176	134	115	185	169	222	74.2	236
	180	655	1408	491	259	209	137	109	187	176	201	74.8	261
	270	398	771	298	180	152	50.2	33.6	39.7	33.9	215	40.1	115
Hair 4	1	141	261	108	70.5	77.4	80.1	55.3	167	233	42.0	38.0	72.6
	10	176	333	136	125	127	104	74.0	192	254	63.8	48.1	109
	20	176	356	143	142	132	117	76.7	189	226	68.8	51.9	126
	30	207	405	156	156	154	127	90.3	196	239	66.5	56.5	141
	45	194	453	152	166	164	132	107	215	238	63.0	52.7	136
	60	293	395	147	154	135	110	86	185	201	73.5	46.6	123
	120	273	534	207	213	171	123	105	214	221	71.4	53.9	183
	180	332	645	225	254	198	143	116	185	171	68.2	62.1	222
	270	291	557	191	229	180	134	96.0	131	140	72.2	46.3	174
Hair 8	1		16.3	<LOQ	29.5	34.0	50.9	35.2	91.9	153	17.7	<LOQ	43.6
	10	<LOQ	25.1	21.2	51.5	50.6	59.9	48.4	103	234	23.4	17.2	59.3
	20	<LOQ	30.7	28.2	59.3	62.6	86.0	59.1	113	167	25.0	17.7	65.3
	30	<LOQ	42.6	24.4	71.1	80.8	115	80.3	178	269	33.3	25.4	83.3
	45	22.4	33.1	13.5	56.7	60.4	90.7	68.8	143	211	26.5	17.4	62.6
	60	174	43.3	31.6	60.7	64.6	113	84.6	156	209	31.4	20.1	71.4
	120	100	83.8	34.3	101	118	140	105	133	152	39.9	28.9	152
	180	43.7	77.7	38.4	99.4	94.7	141	110	156	180	34.8	26.6	91.2
	270	<LOQ	56.6	35.4	70.6	72.5	95.8	72.2	67.0	85.9	21.8	17.8	58.1

*NB: For drugs that were detected, but below the LOQ, results reported as <LOQ. For drugs that were not detected at all, the cell will be greyed out.

3.3.2 Comparison to the overnight method

The conditions chosen that resulted in the best possible extraction within a reasonable time were 2 hours of microwaving at 70 °C. This MAE method was compared to the current 18-hour incubation at 45 °C, using the 3 artificially incorporated samples used in the method development process (labelled 1, 2 and 3), along with the artificially incorporated sample used in Chapter 5 (labelled *Additional*), and authentic hair samples containing drugs of interest (Samples 11 and 12, refer to Chapter 2.2 for details). For the artificially incorporated samples (refer to Table 14) the 2 hour MAE method resulted in higher calculated concentrations for most of the target drugs compared to the 18-hour incubation method. The only analytes that resulted in lower detected concentrations were temazepam, being an average of 13 % lower across hair samples 1, 4 and 8, and oxazepam being an average of 11 % lower across all the artificially incorporated samples. For the additional sample assessed, amphetamine, methamphetamine, diazepam and cocaine also have lower detected concentrations using the MAE method with each of these analytes being 11 %, 2 %, 6 % and 7 % lower than the 18-hour incubation method.

For the authentic hair samples that contain ingested drugs of interest (refer to Table 15) the 2 hour MAE method resulted in similar or higher measured concentrations for most of the target drugs compared to the 18-hour incubation method, indicating the MAE method has the potential to extract more drug from the hair in a shorter period of time. Furthermore, drugs that were not detected in the 18-hour method were detected in the MAE method. Methamphetamine was detected in one of the 3 replicates for the MAE of sample 11 and amphetamine detected in 2 of the 3 replicates for this sample. Oxycodone was also detected (<LOQ) in sample 12 using the MAE, but not with the 18-hour incubation. These drugs detected by MAE were not declared by the donors of the samples. Hence, it may be the result of contamination or carry over from the previous extraction as the microwave extraction tubes were being washed and reused, whereas the 18-hour incubation method used new, disposable tubes each time. The potential for the contamination and carry over to occur is thoroughly investigated in section 4.3.4. The experiments described in that section indicate that carry over is a concern. Despite the potential contamination issue, the MAE method extracted similar amounts of the target analytes, compared to the 18-hour incubation method, which made it a suitable replacement.

Table 14: Calculated concentrations (pg/mg ± 1 SD) from artificially incorporated hair samples using both the MAE method and the 18-hour incubation method for extraction (where n=3)

Sample*	1		4		8		Additional	
	MAE	18 hr	MAE	18 hr	MAE	18 hr	MAE	18 hr
AMP	657 ± 80.5	630 ± 24.8	281 ± 17.2	240 ± 0.706	54.7 ± 30.8	24.4 ± 2.75	986 ± 22.6	1110 ± 18.1
MDMA	1510 ± 227	1370 ± 58.4	585 ± 35.9	469 ± 10.2	73.1 ± 7.12	37.9 ± 2.76	946 ± 23.2	901 ± 116
METH	540 ± 71.8	472 ± 11.7	223 ± 15.3	160 ± 1.74	45.7 ± 22.2	<LOQ	882 ± 35.0	902 ± 55.0
MOR	272 ± 31.7	220 ± 15.0	240 ± 19.0	184 ± 12.2	99.7 ± 6.42	80.3 ± 4.62	398 ± 24.7	352 ± 10.1
COD	228 ± 41.5	200 ± 10.6	211 ± 28.2	161 ± 9.40	112 ± 5.43	87.2 ± 7.21	506 ± 9.77	389 ± 44.2
TEM	159 ± 21.6	228 ± 109	150 ± 19.0	156 ± 20.3	147 ± 7.68	154 ± 26.5	249 ± 59.7	204 ± 24.4
OXAZ	125 ± 17.2	142 ± 10.3	107 ± 2.09	124 ± 11.1	98.9 ± 7.03	115 ± 5.93	282 ± 63.7	295 ± 46.3
NOR	102 ± 58.3	58.0 ± 2.59	117 ± 68.6	57.1 ± 8.65	90.8 ± 27.8	61.2 ± 11.1	85.0 ± 6.50	85.7 ± 12.7
DIAZ	106 ± 44.8	61.6 ± 10.0	132 ± 63.1	72.5 ± 8.51	119 ± 21.2	87.0 ± 7.59	102 ± 9.23	108 ± 14.5
MET	297 ± 58.9	216 ± 23.6	92.4 ± 14.9	69.0 ± 18.3	42.8 ± 6.27	26.3 ± 6.75	165 ± 1.82	151 ± 22.3
COC	75.0 ± 6.56	72.5 ± 3.44	58.6 ± 4.84	48.8 ± 0.494	27.0 ± 1.50	16.8 ± 0.694	100 ± 8.02	107 ± 7.21
OXY	192 ± 36.1	158 ± 32.4	151 ± 23.6	91.6 ± 5.41	96.0 ± 38.5	47.5 ± 6.12	217 ± 9.69	204 ± 5.50

*Each hair sample was exposed to 20,000 pg/mg of hair for each drug

Table 15: Calculated concentrations (pg/mg ± 1 SD) from authentic hairs using both the MAE method and the 18-hour incubation method for extraction (where n=3)

Sample #	11		12	
	MAE	18 hr	MAE	18 hr
AMP	15.8 ± 12.1		128 ± 55.1	143 ± 6.60
MDMA			570 ± 39.8	584 ± 59.1
METH	40.0		322 ± 28.4	262 ± 38.3
MOR			295 ± 31.5	341 ± 84.9
COD	19.6 ± 2.88	<LOQ	61.0 ± 5.35	66.8 ± 14.9
TEM	38.0 ± 5.01	21.8 ± 2.31		
OXAZ	145 ± 6.10	160 ± 10.5		
NOR	<LOQ	<LOQ	<LOQ	<LOQ
DIAZ	<LOQ	<LOQ	<LOQ	<LOQ
MET			95.1 ± 12.4	157 ± 36.9
COC			3840 ± 342	3000 ± 158
OXY	<LOQ	<LOQ	<LOQ	

3.3.3 Validation

A validation study was carried out which investigated linearity, calculated concentration compared to spiked concentration, recovery, matrix effects, and interday precision, and accuracy/ bias (refer to Table 16). The linear range was determined (qualitatively) to be between concentration 1 and concentration 4 (refer to Table 11 for specific concentrations). Morphine, codeine, oxazepam, nordiazepam and diazepam all remained linear up to concentration 5, but the remaining analytes were unable to sustain linearity at those concentrations. Full quantitative evaluation of linearity was not carried out as this parameter relates more to instrument performance than extraction performance; linearity for the instruments has been determined quantitatively as part of a broader validation exercise by FSSA.

Results showed between 80-120 % recovery for most compounds at the 3 concentrations. Temazepam and oxazepam had lower recoveries with oxazepam being the lowest at only 64 % for concentration 3. Methamphetamine and methadone both resulted in very high recoveries for all 3 concentrations. Codeine, nordiazepam and oxycodone also had elevated recoveries. Matrix effects resulted in slight ion enhancement (>100%) throughout the entire data set, except morphine which showed possible ion suppression.

Table 16: Method validation data for MAE of hair at 70 °C for 2 hours (where n=3 where appropriate)

	Linear range (pg/mg)*	Spiked conc. (pg/mg)	Calculated conc. (pg/mg ± SD)	Recovery (%, mean ± SD)	Matrix Effects (%, mean ± SD)	Precision (CV, %)	Accuracy/Bias (%)
AMP	12.5-625	12.5	32.7 ± 27.1	108 ± 4.27	124 ± 5.10	101	-162
		31.3	36.3 ± 6.74	95.6 ± 17.8	120 ± 8.0	22.7	-16.0
		313	317 ± 19.4	87.4 ± 11.3	118 ± 8.27	7.51	-1.51
MDMA	12.5-625	12.5	14.5 ± 0.047	102 ± 4.95	114 ± 11.6	0.399	-15.7
		31.3	29.6 ± 2.95	90.3 ± 13.9	110 ± 13.4	12.2	5.54
		313	331 ± 23.2	94.0 ± 11.0	113 ± 14.1	8.59	-5.99
METH	12.5-625	12.5	114 ± 126	221 ± 51.1	207 ± 26.1	136	-811
		31.3	85.8 ± 55.1	169 ± 32.7	146 ± 15.8	78.6	-174
		313	344 ± 31.7	96.5 ± 11.3	134 ± 12.1	11.3	-10.0
MOR	12.3-613	12.3	12.6 ± 0.283	99.2 ± 2.59	88.9 ± 8.10	2.75	-2.44
		30.6	27.6 ± 2.27	89.6 ± 10.2	86.3 ± 10.2	10.1	9.91
		306	320 ± 36.8	89.4 ± 14.4	87.4 ± 9.76	14.1	-4.58
COD	11.9-594	11.9	18.0 ± 7.01	142 ± 55.6	127 ± 42.4	47.7	-51.3
		29.7	35.4 ± 8.42	114 ± 30.4	111 ± 12.2	29.1	-19.2
		297	298 ± 56.2	80.6 ± 11.7	110 ± 4.83	23.1	-0.20
TEM	26.1-1300	26.1	35.5 ± 2.05	90.2 ± 12.3	106 ± 19.4	7.08	-35.9
		65.1	70.8 ± 14.9	85.7 ± 28.3	116 ±31.2	25.7	-8.76
		651	778 ± 12.4	74.5 ± 2.16	108 ± 27.4	1.95	-19.4
OXAZ	25.6-1280	25.6	25.6 ± 2.57	83.1 ± 11.9	115 ± 26.9	12.3	-0.130
		63.9	50.0 ± 11.6	71.8 ± 23.7	116 ± 31.8	28.5	21.8
		639	574 ± 1.89	63.9 ± 1.68	104 ± 26.5	0.403	10.1
NOR	23.4-1170	23.4	24.1 ± 2.16	119 ± 13.5	174 ± 13.1	11.0	-3.13
		58.4	52.4 ± 6.72	116 ± 24.7	165 ± 12.1	15.7	10.3
		584	615 ± 31.9	112 ± 6.78	170 ± 15.4	6.35	-5.37
DIAZ	25.0-1250	25.0	23.6 ± 0.939	82.4 ± 6.24	126 ± 25.8	4.87	5.47
		62.5	54.6 ± 5.53	79.8 ± 10.7	125 ± 23.7	12.4	12.6
		625	674 ± 53.4	83.4 ± 11.7	123 ± 20.5	9.71	-7.80
MET	11.4-572	11.4	14.9 ± 4.96	143 ± 14.4	151 ± 25.8	40.8	-30.4
		28.6	33.8 ± 14.8	214 ± 71.7	149 ± 25.2	53.7	-18.2
		286	391 ± 77.6	173 ± 26.3	135 ± 22.1	24.3	-36.8
COC	12.5-625	12.5	14.4 ± 1.68	102 ± 16.9	131 ± 27.8	14.2	-15.5
		31.3	30.4 ± 2.90	92.0 ± 13.5	121 ± 20.5	11.7	2.98
		313	334 ± 19.0	96.1 ± 9.68	127 ± 24.3	6.98	-6.83
OXY	10.0-500	10.0	9.30 ± 3.67	117 ± 16.5	111 ± 33.3	48.4	7.00
		25.0	20.8 ± 4.39	112 ± 20.5	114 ± 28.6	25.8	16.7
		250	244 ± 56.3	101 ± 23.9	110 ± 19.7	28.3	2.60

*see Figure 43 and Figure 44 in the Appendix for calibration curves

For accuracy, between ± 15 % was decided to be an acceptable range, which was the case for most compounds at the 3 concentrations. However, a few compounds, notably amphetamine and methamphetamine, exceeded this limit. Amphetamine had poor accuracy for concentration 1 (-162 %) but was improved for the higher concentrations. Methamphetamine had very poor accuracy for both concentration 1 and 2, -811 % and -174 %, respectively, but fell within the acceptable range for the highest concentration. Codeine, temazepam and methadone also had poor accuracies at concentration 1, but to a lesser extent compared to amphetamine and methamphetamine. Methadone had consistently poor accuracy over the concentrations, whereas codeine and temazepam improved. A negative result for the accuracy indicates the detected concentration is higher than the expected (spiked) concentration.

3.3.4 Selectivity and assessment of potential contamination and carryover

The selectivity of the method was assessed by extracting 10 blank hair samples (hair sample 1-10, refer to Chapter 2.2 for details) using the microwave-assisted extraction method at the preferred conditions of 70 °C for 2 hours. Ideally, none of the target analytes would be detected, however, the results presented in Table 17 show that this is not the case. Prior to analysis, the donor of sample 8 (reference number H14-19, refer to Chapter 2.2) declared taking codeine-containing cold and flu tablets, all other donors signed-off on being drug free (i.e. have not knowingly taken any of the target drugs). Hence, it was expected that codeine would be detected in sample 8, but all other samples would be blank. However, methamphetamine was present in quantifiable amounts in 7 of the 10 hair samples, and detected below the limit of quantification for the remaining samples (refer to Table 17). Similarly, amphetamine was present in 8 of the hair samples, but only quantifiable for 8. Diazepam was the only compound not detected in any of the samples. It is likely that some of the more common prescription drugs, such as oxazepam and temazepam, and drugs present in over-the-counter medications such as codeine are actually present in the samples in small quantities but were not declared by the donor. However, it is very unlikely that all of the donors have administered methamphetamine. As such there is potential that contamination has occurred through re-using the microwave extraction tubes.

Table 17: Calculated concentrations (pg/mg) of target analytes in assumed drug-free hair prepared using the MAE method

Sample #	1	2	3	4	5	6	7	8	9	10
AMP	8.33	<LOQ	<LOQ	14.4	<LOQ	<LOQ		1.64	<LOQ	
MDMA							<LOQ		<LOQ	
METH	118	136	173	<LOQ	36.4	26.9	13.9	<LOQ	<LOQ	14.9
MOR					<LOQ					
COD				<LOQ	<LOQ			14.67		
TEM	<LOQ		<LOQ		<LOQ					
OXAZ				<LOQ						
NOR				<LOQ		<LOQ				
DIAZ										
MET	<LOQ									
COC	<LOQ									
OXY				<LOQ	<LOQ					

To ensure this MAE method remained cost effective, the microwave extraction tubes were cleaned, dried and re-used, which may be one possible source of the contamination observed throughout this study. Through microwaving blank methanol in the existing microwave extraction tubes, it was found that amphetamine, MDMA, cocaine and oxycodone were all detected but in levels lower than the limit of quantification. Methamphetamine was detected with concentrations varying from below the level of quantification (<12.5 pg/mg to 48 pg/mg). Prior to these tubes being used for the contamination assessment they were used for the validation study followed by selectivity. This indicates that these drugs, methamphetamine in particular, are lingering in extraction tubes for multiple uses. This could explain the unexpected results for methamphetamine throughout the validation study and why the blank hairs used for the selectivity study contained varying levels of all these compounds. To rectify this, new microwave extraction tubes were purchased and used for the remainder of the study, i.e. the microwave stability study. Due to time constraints, the previous experiments could not be repeated. Prior to using the new microwave tubes, blank methanol was microwaved in three of the tubes and analysed to ensure no target analytes could be detected.

Before beginning this project, other sources of contamination had also been identified, and precautions had been taken. The synthesis of various compounds, including

amphetamine and methamphetamine were also being conducted in the laboratory in which extractions were conducted during a portion of this project. This resulted in large amounts of contamination from the shared glassware and bench space. To reduce contamination from this source separate glassware and work space was used. However, while the additional precautions were taken, and seemed to resolve the issue, the possibility of small amounts of contamination occurring through atmospheric transfer could not be ruled out.

3.3.5 Stability Studies

To assess the stability of each analyte in the microwave during extraction, methanol (2 mL, without hair) was spiked with the target analytes at 3 concentrations, microwaved at the preferred conditions (70 °C for 2 hours) and compared to neat standards of the same concentrations to determine a percentage loss (refer to Figure 7 and Table 31 in the Appendix). Ideally the percent change will be within ±15 %. Within this range, the loss or gain (a negative result indicates an increase in analyte concentration) could be attributed to instrument error, or human error, i.e. lack of precision in spiking the samples. For most of the target analytes the percent loss was within this ±15 % range. Amphetamine and methamphetamine resulted in a slight decline during microwaving at concentration 1, with a loss of 15 % and 17 %, respectively, but greater stability at the higher two concentrations. This phenomenon of the analytes being affected more at lower concentrations is seen for almost all the drugs, with the exception of oxazepam and methadone. Oxazepam had the largest drop in concentration, with an 18 %, 28 % and 25 % loss for concentration 1, 2 and 3 respectively. This is a concern, as this level of loss could result in a false negative, especially when assessing low concentrations, e.g. after a single dose of the drug. Further investigations are needed to determine whether any degradation products are produced from the oxazepam during microwaving, as detection of these degradation products may help to determine whether a false negative has occurred, or if oxazepam was simply not present.

A number of the compounds, including morphine, codeine, temazepam, nordiazepam, diazepam, cocaine and oxycodone, show an increase in the microwaved samples (indicated by the negative values). Except for morphine and codeine, all of these were still between the ± 15 %. Both morphine and codeine had an increase of 23 % for concentration 1. During the validation study, it was found that codeine is subjected to ion

enhancement during analysis (refer to Table 16, Matrix Effects column), which would result in an increase in analyte peak area. However, since this stability study assessed the analyte content in methanol without hair (no matrix), matrix effects is unlikely to have caused this increase. Additional studies are required to understand why these compounds have increased and if this increase would occur if they were spiked to the sample individually, rather than in a mix of drugs.

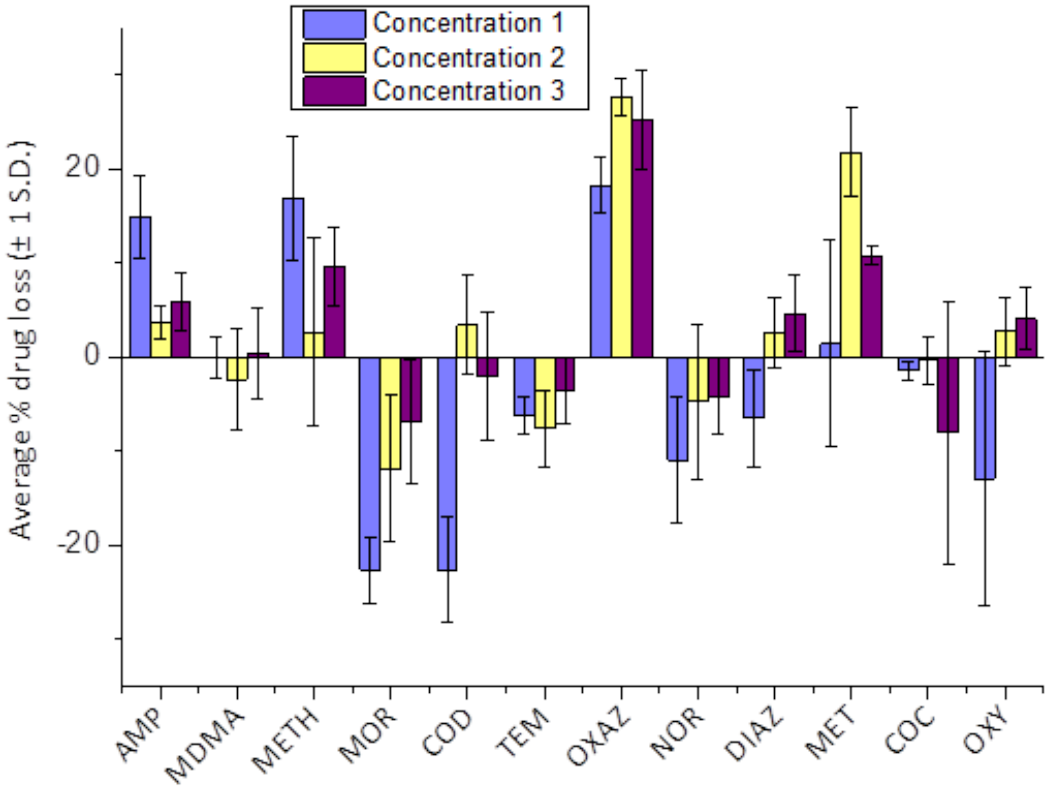


Figure 7: Stability of target analytes after being microwaved (2 hours, 70 °C), showing the percent loss of drug ±1 SD (where n=3). Concentrations for each analyte are as presented in Table 11.

In order to determine extract stability, the extracts from each hair sample at the 3 different concentrations were re-injected 1 day and 1 week after the initial injection, along with the respective neat standards, to assess potential loss of drug and degradation during extract storage. This study is to determine whether a re-injection would give accurate results after having the extract in storage. The percent loss of each analyte at each concentration for both the samples (spiked samples, microwaved with hair) and the neat standards are presented in Table 18. The negative values indicate an increase in analyte rather than a decrease. In general, the percentage loss for each analyte was smaller for the higher concentration, concentration 3, and for most analytes the percent loss was within ±15 %, demonstrating minimal drug loss. The exceptions were temazepam and nordiazepam which both showed losses above 20 % for the samples and neat standards. Temazepam resulted in a loss between 12-24 % after one day, and 24-38 % after 1 week for the sample extract. The neat standard was more stable, resulting in only 2-15 % loss after one day, and 15-28 % after one week. Nordiazepam had the largest decrease, with results indicating a 23-39 % loss after 1 day, and a 49-62 % loss after 1 week, for the sample extract. Similar to temazepam, nordiazepam in the neat standard extract was more stable than in the sample extract, with the maximum loss of 44 %, occurring after 1 week. Hence, it would be reasonable to re-inject the sample to determine an accurate concentration for all of the drugs except temazepam and nordiazepam. Previous validation studies have shown have shown similar results for the stability of drugs in extracts produced via the 18-hour incubation extraction method. Most of the analytes are stable for up to 15 days, with temazepam and nordiazepam showing a significant loss.

Table 18: Stability of target analytes in the final extract (in 0.1 % formic acid in water), showing the percent loss of drug ±1 SD (where n=3) 1 day and 1 week after the initial injection. Results calculated using only the peak area ratios (analyte/IS). Concentrations for each analyte are as presented in Table 11

		Sample		Neat Standard (% loss)	
		% loss (1 day)	% loss (1 week)	% loss (1 day)	% loss (1 week)
AMP	Conc. 1	-5.83 ± 6.87	0.255 ± 6.35	3.26 ± 6.23	1.50 ± 4.70
	Conc. 2	-4.29 ± 3.70	-6.07 ± 10.3	6.85 ± 5.66	3.38 ± 6.11
	Conc. 3	1.66 ± 2.99	-6.47 ± 5.56	-0.290 ± 4.27	-0.290 ± 2.84
MDMA	Conc. 1	4.05 ± 1.12	1.80 ± 5.19	-1.25 ± 1.00	2.25 ± 2.36
	Conc. 2	-0.823 ± 3.58	2.81 ± 5.26	-4.91 ± 4.10	0.0768 ± 1.54
	Conc. 3	-0.304 ± 1.01	-0.623 ± 4.29	0.488 ± 2.10	0.488 ± 3.75
METH	Conc. 1	4.89 ± 1.40	1.74 ± 1.12	8.08 ± 5.76	3.83 ± 3.95
	Conc. 2	8.14 ± 0.442	6.71 ± 0.995	0.569 ± 4.03	2.15 ± 2.60
	Conc. 3	3.60 ± 2.11	0.934 ± 0.335	2.67 ± 3.80	2.67 ± 0.731
MOR	Conc. 1	-3.60 ± 4.08	-0.731 ± 2.90	3.25 ± 4.85	3.73 ± 1.26
	Conc. 2	1.59 ± 2.60	4.69 ± 6.69	0.509 ± 1.60	-2.26 ± 1.67
	Conc. 3	0.231 ± 2.80	-1.08 ± 2.60	-1.95 ± 3.16	-1.95 ± 4.05
COD	Conc. 1	-5.42 ± 11.7	-12.2 ± 7.17	-0.0486 ± 4.84	2.01 ± 2.02
	Conc. 2	3.73 ± 9.28	-0.142 ± 12.0	-6.21 ± 10.4	-9.60 ± 3.98
	Conc. 3	-3.81 ± 7.62	-13.4 ± 14.0	-7.24 ± 3.80	-7.24 ± 6.72
TEM	Conc. 1	23.8 ± 11.6	32.5 ± 16.2	2.15 ± 4.00	28.4 ± 1.89
	Conc. 2	11.6 ± 5.08	38.3 ± 12.0	10.8 ± 3.26	26.9 ± 4.80
	Conc. 3	17.8 ± 8.72	24.2 ± 11.4	14.5 ± 4.06	14.5 ± 3.89
OXAZ	Conc. 1	5.66 ± 19.5	0.0954 ± 12.1	-11.3 ± 4.75	4.29 ± 6.77
	Conc. 2	-3.18 ± 13.8	8.38 ± 23.2	4.89 ± 2.70	4.82 ± 8.77
	Conc. 3	3.76 ± 11.9	0.0482 ± 12.6	3.19 ± 2.91	3.19 ± 4.11
NOR	Conc. 1	39.0 ± 11.0	61.8 ± 2.20	21.6 ± 7.88	40.5 ± 10.5
	Conc. 2	22.8 ± 10.9	48.7 ± 9.13	25.0 ± 2.95	44.0 ± 7.58
	Conc. 3	31.4 ± 2.24	53.9 ± 1.77	24.7 ± 1.07	24.7 ± 7.44
DIAZ	Conc. 1	5.39 ± 8.24	-1.76 ± 19.0	-12.0 ± 9.15	2.68 ± 1.91
	Conc. 2	-1.08 ± 6.70	-2.01 ± 4.06	-0.201 ± 0.587	5.41 ± 3.00
	Conc. 3	-1.29 ± 4.55	-0.208 ± 2.02	1.01 ± 0.989	1.01 ± 3.27
MET	Conc. 1	16.1 ± 7.43	21.1 ± 6.12	4.57 ± 11.3	1.45 ± 23.0
	Conc. 2	1.75 ± 6.46	13.6 ± 3.27	1.35 ± 7.74	13.0 ± 3.65
	Conc. 3	12.3 ± 2.57	31.2 ± 8.80	7.68 ± 5.26	7.68 ± 3.51
COC	Conc. 1	-1.92 ± 4.10	-3.06 ± 1.99	-0.728 ± 2.77	-0.651 ± 0.552
	Conc. 2	-0.369 ± 2.66	-3.79 ± 2.38	-0.688 ± 2.29	-2.40 ± 1.80
	Conc. 3	-0.349 ± 2.47	-3.57 ± 1.88	-6.34 ± 2.57	-6.34 ± 3.16
OXY	Conc. 1	7.91 ± 15.4	7.14 ± 8.92	-6.83 ± 7.28	4.88 ± 11.9
	Conc. 2	15.3 ± 9.36	9.70 ± 8.51	1.54 ± 5.32	6.33 ± 13.2
	Conc. 3	14.4 ± 9.69	11.1 ± 9.10	-1.66 ± 4.47	-1.66 ± 10.3

3.4 Conclusions

This chapter investigated the potential of using microwave heating as an alternative to the standard overnight incubation extraction method. The MAE method, presented in this chapter, of microwaving finely-cut hair in methanol (20 mg: 2 mL) for 2 hours at 70 °C provides a fast and effective way of extracting the 12 target analytes assessed (of which included 3 analytes from a drug class that has not previously been investigated in this way), without hindering recovery. This method extracted similar amounts of the target analytes, compared to the 18-hour incubation method, which made it a suitable replacement. Additional studies may be performed to better optimize the MAE method, such as ball-milling or pulverization of the hair prior to microwaving, use of alternative solvents and assessment of additional microwave conditions, i.e. power output. However, for this method to be effective, a multi-vessel microwave will need to be used, rather than the single vessel microwave used for this study. Additionally, due to issues with contamination, if the microwave tubes are to be re-used, a more thorough cleaning method will need to be developed and used to ensure no carry-over of target analytes between uses. If a more reliable cleaning method cannot be employed, the extractions will need to be conducted in a new extraction tube each time.

Chapter 4

The use of an ionic liquid composite to disassemble hair

This chapter examines the use of the ionic liquid composite (ILC) choline chloride: urea (1:2 molar ratio) in combination with microwave irradiation to disassemble the structure of hair to allow for rapid extraction of drugs for analysis. Results discussed in this chapter include:

- *Disassembling hair in the ILC using conventional heating*
- *Method development for disassembling hair in the ILC using microwave irradiation, including variation in microwave time, temperature, and power outputs, and the optimal volume of ILC to hair ratio*
- *Assessment of extraction and clean-up methods*
- *Recovery and stability of target analytes throughout microwaving process*
- *Preliminary studies/future work: disassembling hair in the ILC using microwave irradiation for detection of heavy metals in hair using ICPMS analysis*

4.1 Introduction

An ionic liquid (IL) is a salt that is a liquid at temperatures below 100°C. Due to their unusual properties IL's are quickly becoming popular in all areas of chemistry as they typically have low volatility, good thermal stability, high viscosity, poor conduction of electricity, cost effective and favourable environmental properties [155]. Ionic liquid composites (ILC), also known as deep eutectic solvents, are solvents where the liquid comprises a mixture of ionic and neutral molecules that form a eutectic with a melting point substantially lower than that of the separate constituents. Deep eutectics occur due of the formation of complex anions, reducing lattice energy, and hence decreasing the melting point of the system. Abbott *et al.* [156] reported that a urea/choline chloride composite forms a deep eutectic at a 2:1 molar ratio. This particular ILC remains in liquid state to 12 °C, whereas the individual melting points of choline chloride and urea are 302 °C and 133 °C, respectively. A study by Boulos *et al.* [6] recently used this mixture of choline chloride and urea to partly disassemble the keratinized structure of hair into functional materials at room temperature. They showed that, at room temperature, treatment of hair with urea/choline chloride ILC shears off the outer cuticle layer of the hair shaft, yet much of the hair shaft remains intact. Unpublished work by Boulos *et al.* demonstrated that dissolution of the entire hair shaft occurs when the ILC is heated to 150°C. It has been reported that ionic liquids such as these could be used for forensic applications (such as extracting genetic material) [157], analytical techniques [158, 159], drug delivery purposes, producing components for sunscreens, shampoos and dietary supplements [160], recycling of cellulose and keratin for environmental purposes [161, 162], use in sensor design [163, 164]. While ionic liquids (IL) and ionic liquid composites (ILC) are very versatile and are used extensively in analytical chemistry, there is no prior work published on the use of ionic liquids for digestion and extraction of drugs from hair.

We hypothesised that a choline chloride/urea composite similar to that reported by Boulos *et al.* could be used as an alternative to the traditional method of hair sample preparation which use ball milling or cutting in combination with organic solvent extraction. Firstly, the ILC would disassemble the hair structure, exposing the drugs of interest. Secondly the target analyte could be extracted from the ILC hair digest using an extraction procedure such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE). One key property of this urea/choline chloride ILC is that it is water soluble, which would

allow for better extraction than other water insoluble ILCs. Digestion at room temperature, as reported by Boulos *et al.* [6], was considered unsuitable as much of the hair remains intact, potentially leaving behind a significant amount of target analyte still trapped within the hair. Simply elevating the temperature to 150°C to achieve full dissolution was considered non-ideal as a number of drugs of abuse or other potential target analytes such as benzodiazepines and amphetamines are thermally unstable at this temperature. A commonly used method digests the hair with aqueous 1M NaOH for 1 hour at 80°C, and still provides high yields for cannabinoids or amphetamines, nicotine, antidepressants and neuroleptics, as well as several other drugs (all alkaline-stable) [68]. This provides a guide for how high the IL composite could be heated (and for how long) before it begins to compromise the stability of the drugs. Ideally short extraction times (less than 1 hour) and temperatures less than 80°C are required to minimise thermal degradation.

Microwave-assisted extraction (MAE) uses microwave energy to rapidly heat solvent/sample mixtures. It is known to accelerate the mass transfer of target compounds from the sample matrix into the solvent, with extractions being able to be performed with less solvent and in a shorter timeframe. In most cases analyte recovery and reproducibility are improved compared to conventional techniques. Microwave-assisted digestion may be a way to accelerate the digestion of hair in the ILC as well as reduce the temperature at which the digestion occurs. A major benefit of using microwave digestion over traditional heating is the ability to rapidly heat and, with the aid of in-built cooling systems, to rapidly cool the sample. This has the potential to drastically reduce the time the sample and the analytes within are exposed to high temperatures, and thus minimise the potential for thermal degradation.

This chapter examines the use of the ionic liquid composite (ILC) urea/choline chloride (2:1 molar ratio) in combination with microwave assisted digestion to disassemble the structure of hair, followed by a liquid-liquid extraction method to extract target analytes for analysis. The aim was to develop a fast and effective method of disassembling the hair and extracting target analytes at conditions that will not result in drug degradation and is either faster than the current method used whilst still maintaining similar drug recoveries. Reduced extraction times will greatly improve case turnaround times and reduced case back-log currently experienced at Forensic Science SA (the industry partners for this

project). However, due to the ILC needing to be heated to a high temperature for dissolution to occur and the viscosity of the ILC, extraction and detection of drugs hair using this method was difficult and resulted in loss of drug. This chapter also presents preliminary work into the development of an alternative method for preparing hair samples for ICP-MS analysis using the urea/choline chloride composite.

4.2 Experimental

4.2.1 Hair sample collection and preparation

Hair samples from different donors were collected and screened prior to use to ensure all samples were drug-free. These drug-free hair samples (20 mg per sample, finely cut into 1-5 mm fragments) were washed with methanol (2 mL) and left to dry at room temperature prior to use. For the method development of the microwave digestion and extraction (including assessment of mass restricted filters) hair sample 7 (refer to Table 4, Chapter 2.2), was used due to large quantity provided. For the drug stability and drug recovery studies hair samples 1, 4 and 8 (refer to Table 4, Chapter 2.2) were used due to quantities provided and variation in colour and thickness between hair samples.

4.2.2 Preparation of ILC

The ILC was prepared according to Boulos *et al.* [6] who used urea/choline chloride (2:1 molar). The 2:1 molar mix was prepared by heating to 150 °C and mixing for 1 hour. The ILC was allowed to cool before use.

4.2.3 Disassembling the structure of hair with ILC using conventional heating

The ILC was mixed with hair (2mL: 20mg) at room temperature, 50 °C, 100 °C and 150 °C. Mixtures were held at these temperatures for periods of 30 minutes, 1 hour, 5 hours and overnight. The ILC: hair mixtures were not exposed to higher temperatures (100 °C and 150 °C) for periods longer than 5 hours. The ILC and hair (2mL: 20mg) were ultra-sonicated together for 30 minutes (at room temperature).

4.2.4 Disassembling the structure of hair using microwave assisted digestion

A microwave-assisted digestion method was developed by assessing different microwave conditions to allow for 20 mg of hair to undergo full or partial dissolution. Preliminary experiments assessed the ILC:hair ratio (2mL:20mg, 1.5mL:20mg, 1mL:20mg

0.75mL:20mg, 0.5mL:20mg). The microwave conditions of focus included power output (20 W-70 W, at 10 W intervals), and temperature settings (60 °C-100 °C, at 5 °C intervals). Due to concerns of analyte degradation, the set microwave temperature did not exceed 100 °C.

4.2.5 ILC Extraction and Clean up

Extraction and clean-up was assessed to prepare digested hair samples for analysis. The final extraction method used an ammonium hydroxide (35 %w/v) solution as the buffer and *n*-butyl chloride as the extraction solvent based on the Screening for Basic Drugs method employed at Forensic Science South Australia [165]. Milli-Q water (1.5 mL) was added to the ILC sample containing digested hair (0.5 mL) and the pH of ILC mix (i.e. ILC sample containing digested hair and the Milli-Q water) was adjusted with the ammonia buffer (0.025 mL). The pH was then measured to ensure it was a pH between 10 and 12 to enable effective extraction of basic drugs into the organic solvent. The resulting solution was mixed with *n*-butyl chloride (10 mL). This was then followed by centrifugation (40 minutes at 3000 rpm) and the collection of the organic solvent, which was evaporated down and reconstituted in 0.1 % formic acid buffer (100 µL) for injection into the LC-QTOF instrument. Internal standard (20 µL) was added to the *n*-butyl chloride immediately before evaporation. All samples were analysed using the Agilent LC-QTOF (refer to Chapter 2.3 for details).

During the initial assessment of the extraction procedures, only 5 mg hair was digested in 0.25 mL ILC and extracted with the above method, using D.I. water (0.5 mL), ammonia buffer (0.025 mL), *n*-BuCl (2 mL). Smaller volumes/quantities were used initially to ensure residues from the ILC and digested hair would not block or damage the column used in chromatographic separation and detection, and to determine if there would be any major interferences within the matrix. To optimise extraction, varying water and butyl chloride volumes (0.5 mL, 0.75 mL and 1 mL H_20, and 2 mL and 5 mL *n*-BuCl) and the use of mass restricted centrifugal filters were assessed. The volume ratios and mass restricted filters were also investigated using 5 mg hair and 0.25 mL ILC.

Mass Restricted Centrifuge Filters

Four aliquots of hair were microwaved in ILC (5 mg: 0.25 mL, 60 °C, 60 W). All four samples, plus two blank (hair-free) ILC samples (0.25 mL) were spiked with drug mix

(10 µL, 1 µg/mL) containing morphine, codeine, amphetamine, methamphetamine, MDMA, methadone, cocaine and oxycodone (benzodiazepines not included in this mix) once cooled to room temperature. The two hair-free ILC samples and two of the microwave hair samples were extracted using the liquid-liquid extraction method. The remaining two samples were diluted using D.I. water (0.5 mL) transferred and centrifuged through mass restricted filters (Microsep™ Advanced Centrifugal Device, 10 KD) for 40 minutes at 3000 rpm. From here 2 separate methods were used for each sample. For sample A) The ammonium buffer was added to the filtered ILC samples, followed by the *n*-BuCl (5 mL); B) The ammonium buffer was added to the filtered ILC samples, followed by 4 mL of the *n*-BuCl, an additional 1 mL of *n*-BuCl was centrifuged through the filter as a wash. All samples were then vortexed and continued the LLE process stated above. The recovery of each analyte was determined by calculating the peak area ratio (standard/IS) of the extracted sample and dividing by the peak area ratio (standard/IS) of the neat standard, and multiplying by 100 to obtain a percent.

4.2.6 Drug stability and recovery

Drug stability

A standard drug mix (DMIX) containing morphine, codeine, amphetamine, methamphetamine, MDMA, methadone, cocaine, oxycodone, diazepam, nordiazepam, oxazepam and temazepam was prepared by ethanol dilution at 1 µg/mL. The drug mix (10 µL) was spiked directly to the ILC/hair sample (0.5 mL: 20 mg) prior to microwaving at the optimised conditions, and then extracted using the LLE method above. These samples were then compared to samples that were spiked post microwave (i.e. the drug mix did not go above room temperature). The stability of each analyte was determined by calculating according to Equation 6 (refer to Chapter 3.2.5).

Drug recovery

Recovery using the optimised ILC microwave digestion method was compared to the current 18-hour methanol solvent extraction at 45 °C. Recovery was assessed using hair samples artificially incorporated with drugs (refer to Chapter 2.2, for method of artificial drug incorporation into hair). The recovery of each analyte was determined by calculating the peak area ratio (analyte/IS) of the microwaved samples and dividing by the peak area

ratio (analyte/IS) of the samples extracted via the overnight incubation (18 hours, 45 °C) in methanol (2 mL), and multiplying by 100 to obtain a percent.

4.2.7 Future work (heavy metals in hair)

Another type of forensically relevant analyte that can be detected in hair is heavy metals. Traditionally, the sample preparation methods for analysis of heavy metals in hair involve either dry ashing, or heated acid digestion. These processes can be lengthy and rather dangerous. Detection of heavy metals in hair through the use of ILC digestion was briefly investigated as an alternative use for the ionic liquid composite digestion method. This was accomplished by digesting 20.8 mg of cleaned hair (hair sample 6, refer to Table 4, Chapter 2.2) in 0.5 mL ILC to completion, at 150 °C. The digested hair in the ILC was then diluted by 1 in 1000 and 1 in 100 in MilliQ water, and acidified to 2% HNO_3/HCl prior to analysis by ICP-MS (refer to Chapter 2.4.6). A blank ILC sample was also assessed at these dilution levels to ensure there was no contamination of heavy metals in the choline chloride or urea starting products. The heavy metals assessed were copper, zinc, arsenic, cadmium, mercury and lead. The hair donor had not been knowingly exposed to high levels of any of these selected analytes. Internal standards were also monitored throughout the analysis as a way of monitoring potential matrix effects.

4.4 Results and Discussion

4.4.1 Preliminary studies

The extent to which the hair can be disassembled by the ILC was assessed by mixing 20 mg of hair in 2 mL of the ILC with conventional heating at room temperature, 50 °C, 100 °C and 150 °C for 30 minutes, 1 hour and 5 hours at all temperatures, and overnight at room temperature and 50 °C. Previous studies reported by Boulos *et al.* state the ILC will remove the cuticle scales when mixed with hair at room temperature overnight, however this was not observed in this study. There was no change to the hair structure after mixing at room temperature regardless of time. At 50 °C there was a slight change, with the removal of some of the cuticle cells, but only after heating overnight. It was observed that by heating the hair and ILC to 100 °C, the cuticle scales could be removed. After 1 hour there was an inconsistent degree of removal which can be seen in Figure 8 and Figure 9. The treated hairs in both figures were taken from the same donor and sample after being heated at 100 °C for 1 hour. Figure 9 shows the complete removal of the cuticle scales,

whereas Figure 8 shows the cuticle scales still intact. By increasing the temperature to 150 °C, the hair completely dissolved (refer to Figure 10) within 10 minutes. However, high temperatures could affect drug stability and recovery, so an alternative method of heating was needed to assist in the digestion process. Ultra-sonication for 1 hour was trialled. This resulted in no change to the structure of hair. Microwave heating was then employed to assist with the digestion.

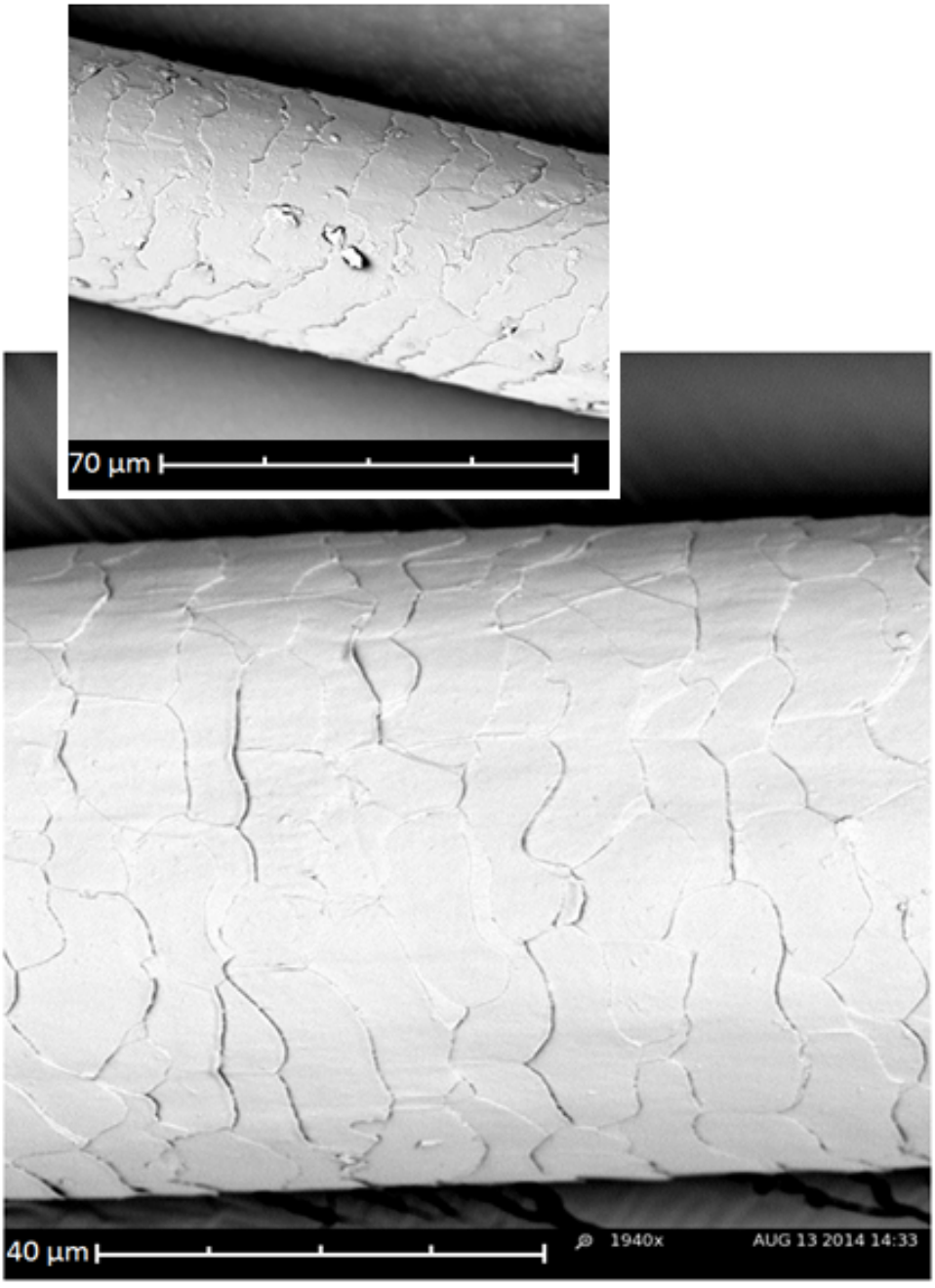


Figure 8: SEM images of untreated donor hair (top), compared to hair from the same donor being treated using the ILC, mixed for 1 hour at 100 °C (bottom). Images taken using Inspect FEI F50 SEM.

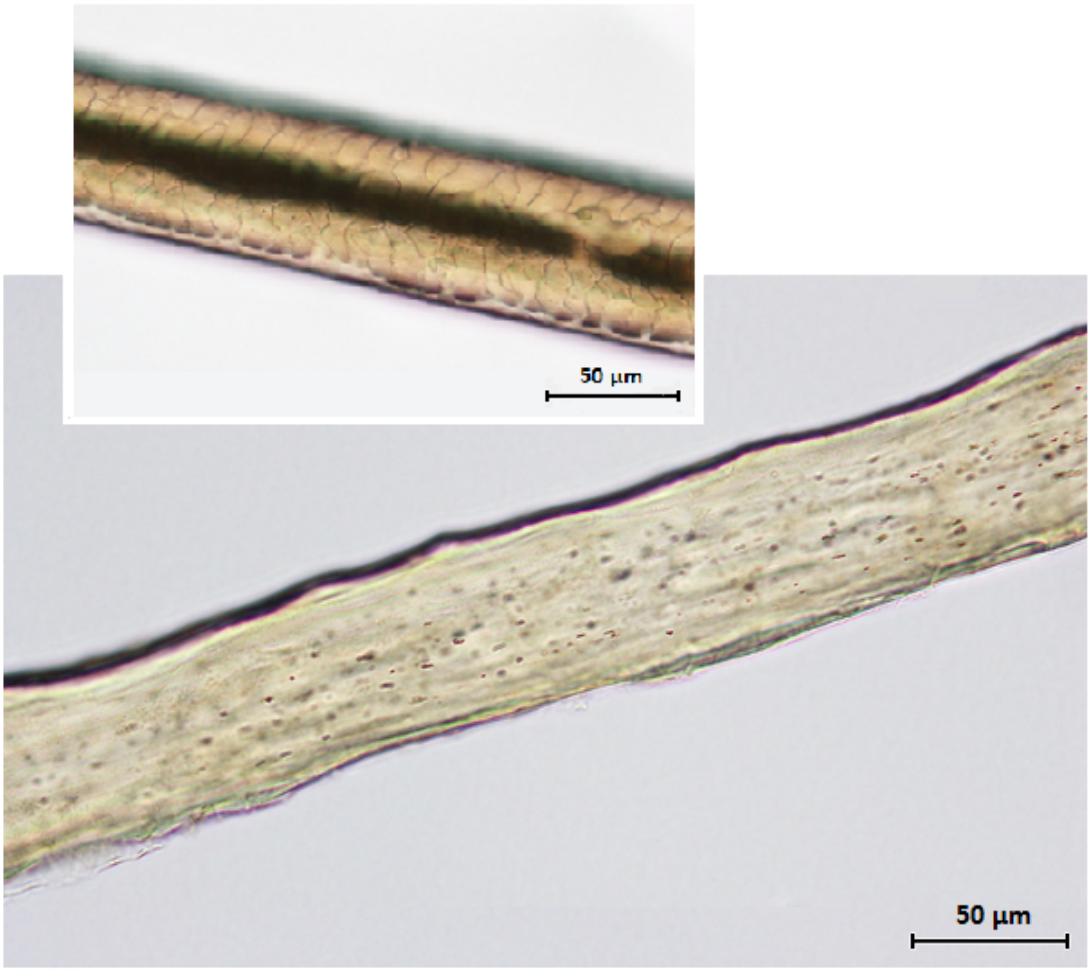


Figure 9: Transmitted light microscopy images of hair treated using the ILC for 1 hour at 100 °C (bottom) compared with untreated hair from the same donor (top). Images taken an Olympus CX41 Compound microscope. Noteworthy is the complete absence of scales in the treated hair. The absence of a medulla in the treated hair is not a result of the treatment, this particular hair did not have a medulla before treatment.

Figure 10: 20 mg of un-cut hair in 2 mL ILC, before heat treatment (left); 20 mg of un-cut hair in 2 mL ILC after being heated in an oil bath to 150 °C (right)

4.4.2 Microwave digestion

Microwave irradiation was used in place of regular heating to enable dissolution of hair in the ILC at lower temperatures. The microwave reactor enables rapid heating and rapid cooling which was found to speed up the dissolution process. Figure 11 shows the combination of the ILC and microwave irradiation results in a much faster and effective digestion method. At 100 °C in oil bath for 1 hour the effect on the hair is minimal (refer to Figure 8 and Figure 9), however with the assistance of microwave irradiation the ILC has completely removed the outer layer of cuticle scales, and has begun to penetrate and break down the cortex after only 2 minutes of heating (total time taken to heat to 100 °C and cool to room temperature). It is unclear as to whether the hair needs to be completely dissolved to enable release of drugs within the hair.

During preliminary microwave experiments, it was found the optimum ILC volume to hair ratio was 0.5 mL: 20 mg, as 0.5 mL is enough to completely immerse the 20 mg of hair, whilst using minimal quantities of ILC. A smaller quantity of ILC is desirable as it will enable easier extraction. Using larger volumes of ILC also resulted in dissolution taking longer and needing higher temperatures.

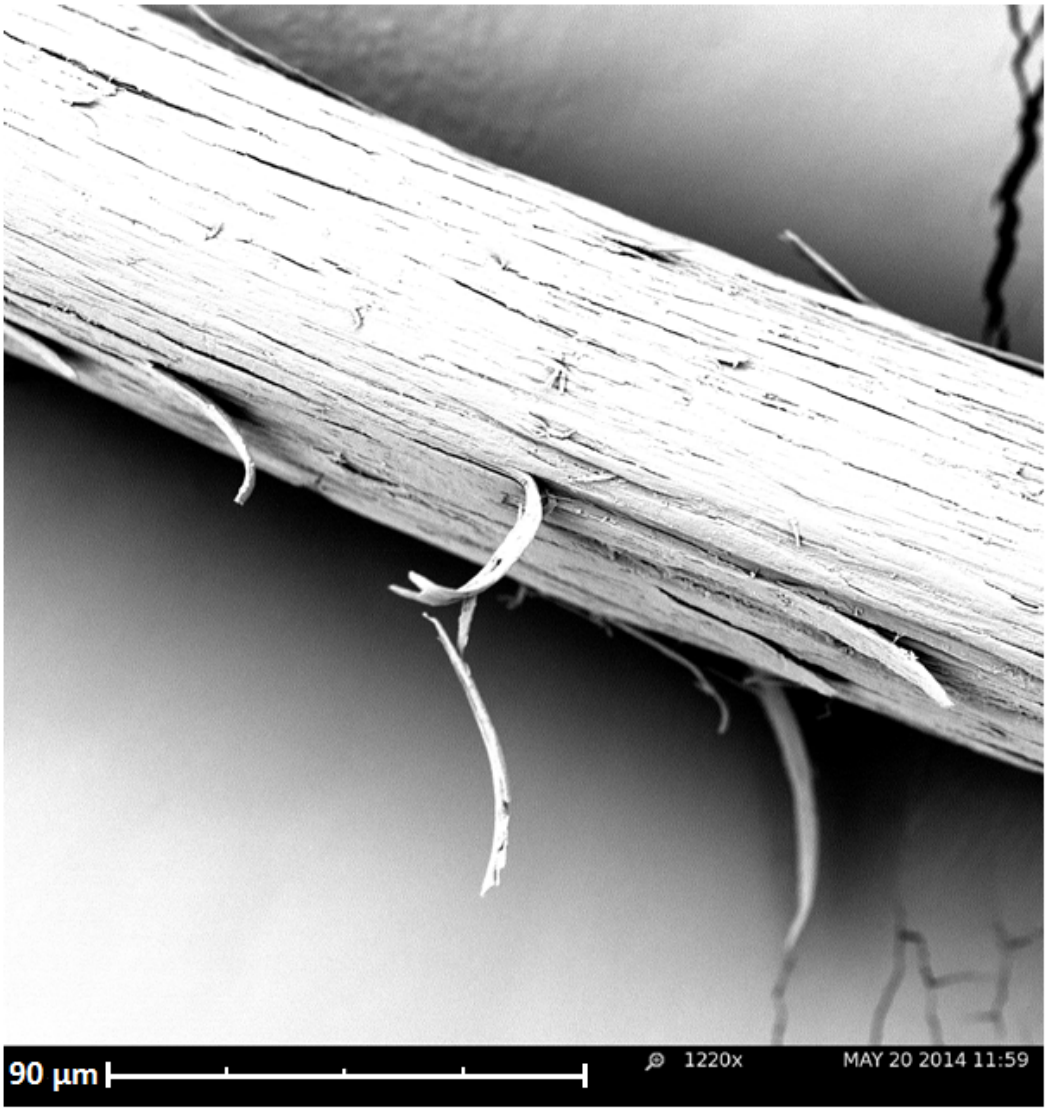


Figure 11: SEM image of hair after treatment with ILC using microwave digestion (partially dissolved hair) at 100 °C for 2 minutes (including heating and cooling periods). Image taken using Inspect FEI F50 SEM

One benefit of using a microwave reactor is it enables the user to monitor and maintain a consistent temperature for a set time before cooling the sample by bombarding the vessel with compressed air for rapid cooling. Unfortunately, during these preliminary trials it was found that even after the microwave stopped heating, the sample would continue increasing in temperature, i.e. the microwave would be set to reach 100 °C and hold that temperature for 1-2 minutes, but was actually reaching temperatures of up to 150 °C. Hence for the remainder of the study the microwave was set to cool immediately once it hit the set temperature. Even still, the sample continued to heat during the cooling

period. For this reason each sample was microwaved twice in order to achieve complete dissolution without exposing the sample to excessive temperatures.

To maximise dissolution, whilst keeping the temperature as low as possible, the power output and set temperature settings were assessed. As mentioned previously the temperature the sample reached always exceeded the set temperature, hence the maximum temperature reached for each sample was recorded, which can be seen in Table 19.

Table 19: Average temperature reached (± 1 SD) for set conditions, where both power output and temperature was assessed (where n=3 donor's hair samples, microwaved twice each)

	Temperature reached (°C)			
Set conditions	40W	50W	60W	70W
100 °C	108 ± 3.54	119 ± 4.95	124 ± 3.54	124 ± 7.23
95 °C	114 ± 0.707	120 ± 0.707	123 ± 4.20	124 ± 3.51
90 °C	115 ± 0.707	117 ± 2.12	118 ± 3.10	124 ±3.54
85 °C	104 ± 4.95	120 ± 5.66	127 ± 4.51	124 ±2.31
80 °C	103 ± 4.24	120 ± 5.66	121 ± 4.76	115 ± 8.08
75 °C	108 ± 0.707	104 ± 4.95	121 ± 4.40	113 ± 11.6
70 °C	105 ± 1.41	105 ± 4.95	112 ± 5.91	119 ± 2.00
65 °C	93.0 ± 5.66	103 ± 4.24	112 ± 6.95	101 ± 5.12
60 °C	98.5 ± 2.12	95.0 ± 9.90	105 ± 2.31	105 ± 5.80

Using 40 and 50 W, very little dissolution occurred, even at the higher temperatures. As was the same for anything below 60 °C (not recorded in the table), where very little, if any, dissolution occurred but the temperature of the sample was still reaching above 80 °C. At 60 W the level of dissolution was more consistent between samples than at 70 W. When set to 100°C at 60W, every sample underwent complete dissolution after only one heating. This may be beneficial for the drug stability as the sample is only exposed to the potentially damaging high temperatures once, and for a very short duration. This was the only combination of conditions which allowed for complete dissolution consistently. However, the temperature reached was believed to be too high. Between 60-70 °C at 60 W, the level of dissolution was considered sufficient enough to continue to the next stage of testing. These conditions were assessed on the remaining

hair samples #2-10 (refer to Chapter 2.2). The average maximum temperature reached and percent of hair remaining after microwaving is presented in Table 20.

Table 20: The average maximum temperature reached (± 1 SD) across the different hair samples and the percent mass of undissolved hair remaining (± 1 SD) after microwaving (where n=9)

	60 °C	65 °C	70 °C
Temp. reached (°C)	105.5 ± 5.28	112 ± 5.35	114 ± 3.21
% hair remaining	41.9 ± 13.3	34.7 ± 18.2	20.9 ± 15.8

At the lowest possible temperature setting at which some level of dissolution occurs, the temperature the samples were reaching (105-114 °C) is well above what would be considered suitable for drug extraction. However, these conditions were used to assess recovery and drug stability, since using conventional heating, even though can be done at lower temperatures, takes even longer than the current method of sample preparation.

4.4.3 Extraction and Clean up

Through the initial assessment of the extraction, i.e. hair free ILC extracted using the LLE method, a large interference with *m/z* of 104.1080 was found (refer to Figure 12). Agilent MassHunter software predicted the chemical formula to be $C_5H_{13}NO$ (δ=-9.7 ppm, -1.1 mDa) which corresponds to choline, a component of the ILC. This interference, although eluting throughout the entire run, is most prominent in the first 2 minutes, which could result in high levels of ion suppression or enhancement for the early eluting compounds, such as morphine, codeine, amphetamine and oxycodone, which have retention times of 1.93, 2.07, 2.75, and 2.88 minutes, respectively.

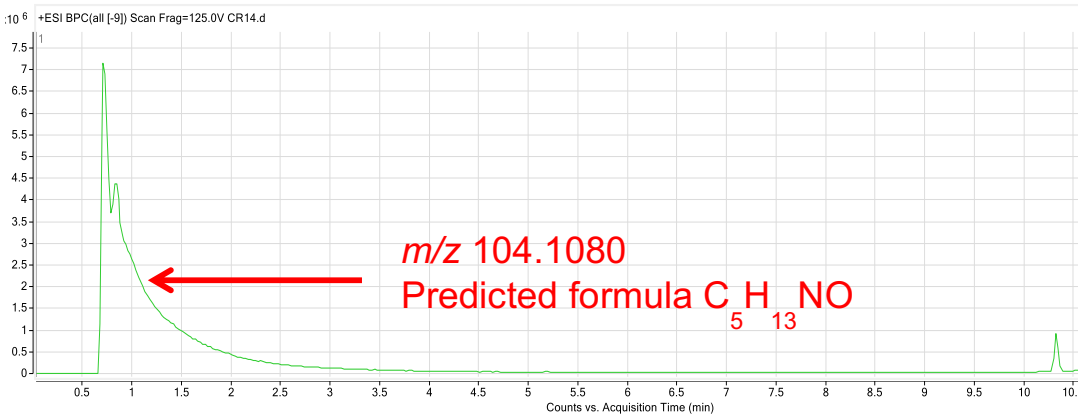


Figure 12: Chromatogram of extracted hair digested in ILC (no drugs present). Green signal showing the intensity of choline in the early stages of the chromatogram

Another issue encountered with regard to extraction and clean-up was the high viscosity of the ILC. This made transferring the sample difficult, as well as hindering the LLE procedure. Initially, only 0.5 mL H_2O and 2 mL *n*-BuCl was used per 0.25 mL ILC. These small volumes were not enough to separate the ILC into the aqueous layer and hence an emulsion occurred. Various H_2O and *n*-BuCl volumes were trialled (refer to Table 21). By increasing the solvent volumes the organic and aqueous layers separate out properly and no longer form the emulsion, the drug recovery is improved, and a significant reduction in the 104 m/z interfering peak was also observed. There were still a number of interfering peaks present in the samples, but were less abundant. The final volumes used for the extraction and clean-up process were 0.75 mL H_2O, 5 mL *n*-BuCl for 0.25 mL ILC. Since the microwave hair digestion method uses 0.5 mL ILC with 20 mg hair, the water and butyl chloride volumes were also adjusted accordingly, i.e. increased to 1.5 mL and 10 mL respectively. The increase in solvent volume, however, means that the extraction cannot be carried out in the 10 mL capacity microwave tube, requiring a tube change, compromising the efficiency of the extraction process.

Table 21: Assessment of varying solvent ratios for LLE, results present as % recovery (±1 SD)

	2 mL n-BuCl			5 mL n-BuCl		
	0.5 mL H_20	0.75 mL H_20	1 mL H_20	0.5 mL H_20	0.75 mL H_20	1 mL H_20
AMP	10.1 ± 3.33	6.88 ± 0.731	8.15 ± 2.35	17.6 ± 4.68	15.1 ± 4.86	9.26 ± 0.662
MDMA	13.1 ± 3.65	9.68 ± 0.476	9.53 ± 1.12	24.6 ± 3.94	22.1 ± 1.88	16.6 ± 2.95
METH	38.4 ± 3.20	44.2 ± 11.5	35.7 ± 4.26	47.9 ± 62.1	44.8 ± 0.921	45.6 ± 10.6
MOR	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
COD	44.6 ± 3.99	41.1 ± 2.47	35.0 ± 1.33	78.4 ± 12.9	84.1 ± 1.76	76.7 ± 17.9
TEM*						
OXAZ*						
NOR*						
DIAZ*						
MET	58.9 ± 19.4	42.1 ± 3.84	37.9 ± 7.69	81.0 ± 15.1	75.2 ± 4.83	79.9 ± 25.9
COC	12.3 ± 3.40	20.8 ± 2.70	14.3 ± 5.14	50.4 ± 19.7	44.8 ± 14.0	19.8 ± 7.60
OXY	21.8 ± 7.22	28.9 ± 8.88	20.1 ± 6.54	76.7 ± 65.3	52.6 ± 7.90	24.9 ±16.5

*Analytes not tested as the laboratory did not have permits for these drugs at the time of the experiment.

It should be noted that at this stage of the evaluation, morphine was not being detected due to this LLE method being designed to extract basic drugs, whereas morphine is amphoteric, i.e. contains both acidic and basic groups. The effectiveness of a LLE method is primarily dependant on the pK_a of the target analyte compared to the pH of the solution. For the best extraction of an analyte from an aqueous phase into an organic layer, the compounds should be completely de-ionised. For a basic compound, almost complete de-protonation (de-ionisation) will occur when the extraction pH is approximately 2 units above the pK_a of the analyte. Similarly, for an acidic compound, virtually complete protonation (de-ionisation) will occur when the extraction pH is approximately 2 units below the pK_a of the analyte [166]. This is beneficial when trying to extract multiple drugs of the same type i.e. acid or base. It becomes an issue when trying to simultaneously extract drugs from both the acid and base categories, including amphoteric compounds, such as morphine. For the detection of drugs such as morphine, alternative extraction methods are needed.

Mass restricted filters

To assist with the clean-up process and to further reduce interfering peaks, Microsep™ Advanced Centrifugal 10 KD mass restricted devices were assessed. Samples were provided by Stennick Scientific, giving only 2 cartridges to complete preliminary experiments. The purpose of these filters was to remove any undissolved hair from the ILC and to reduce high molecular weight substances that might interfere with the chromatographic analysis. In actual fact, using the mass restricted filters hindered the extraction process further, producing more interferences and resulting in less drug recovered compared to the original LLE method that does not use the filters.

The extracts produced were visually darker in appearance than those prepared through the standard LLE method resulting in larger potentially interfering peaks (refer to Figure 13). The blue signal in both the base peak chromatograms (BPC), representing the neat drug standard made up in the 0.1 % formic acid mobile phase, has little to no background noise or interference. This, ideally, is the standard the extracted samples should be meeting. However, samples extracted with the assistance of the filters produced large interfering peaks (green and red signals in the top chromatogram) compared to both the neat standard and the samples extracted using the standard LLE method. Even though the standard LLE method produces relatively large interfering peaks (pink and black signals present in bottom chromatogram), it is significantly less than the filter method. The increase in interfering peaks may be due to components from the filter cartridge leaching through into the extract.

Additionally, the cartridge became clogged early on in the filtration process and not all of the ILC was able to filter through the cartridge. Even using the smaller ILC and hair quantities (0.25 mL:5 mg), additional centrifuging and the addition of *n*-BuCl to 'flush' the cartridge was not enough to allow all of the desired material to pass through. This may be why the drug recoveries (refer to Table 22) were lower than if no filter was used for MDMA, codeine, methadone, cocaine and oxycodone. Using the organic solvent as a wash through the filter (filter method B) did result in an increase in recovery, especially for methadone, cocaine and oxycodone. If further experiments were to be completed using the centrifugal filtration devices, a more extensive solvent wash would be recommended.

The standard LLE method, both with and without hair, still had low drug recoveries. This could potentially be loss due the tube change, issues with the viscosity, or just an inefficient extraction method. Using alternative extraction methods such as solid-phase extraction (SPE) would not be recommended as the ILC is likely to clog the SPE cartridge in a similar manner to the centrifugal cartridges. Further investigation into alternative liquid-liquid extraction methods, i.e. different extraction solvents, could improve the recovery of target analytes and reduce interferences. However, before trialling alternative extraction methods, the drug stability and recovery from incorporated hairs using the microwave ILC digestion process needed to be assessed in order to verify whether it was necessary to continue.

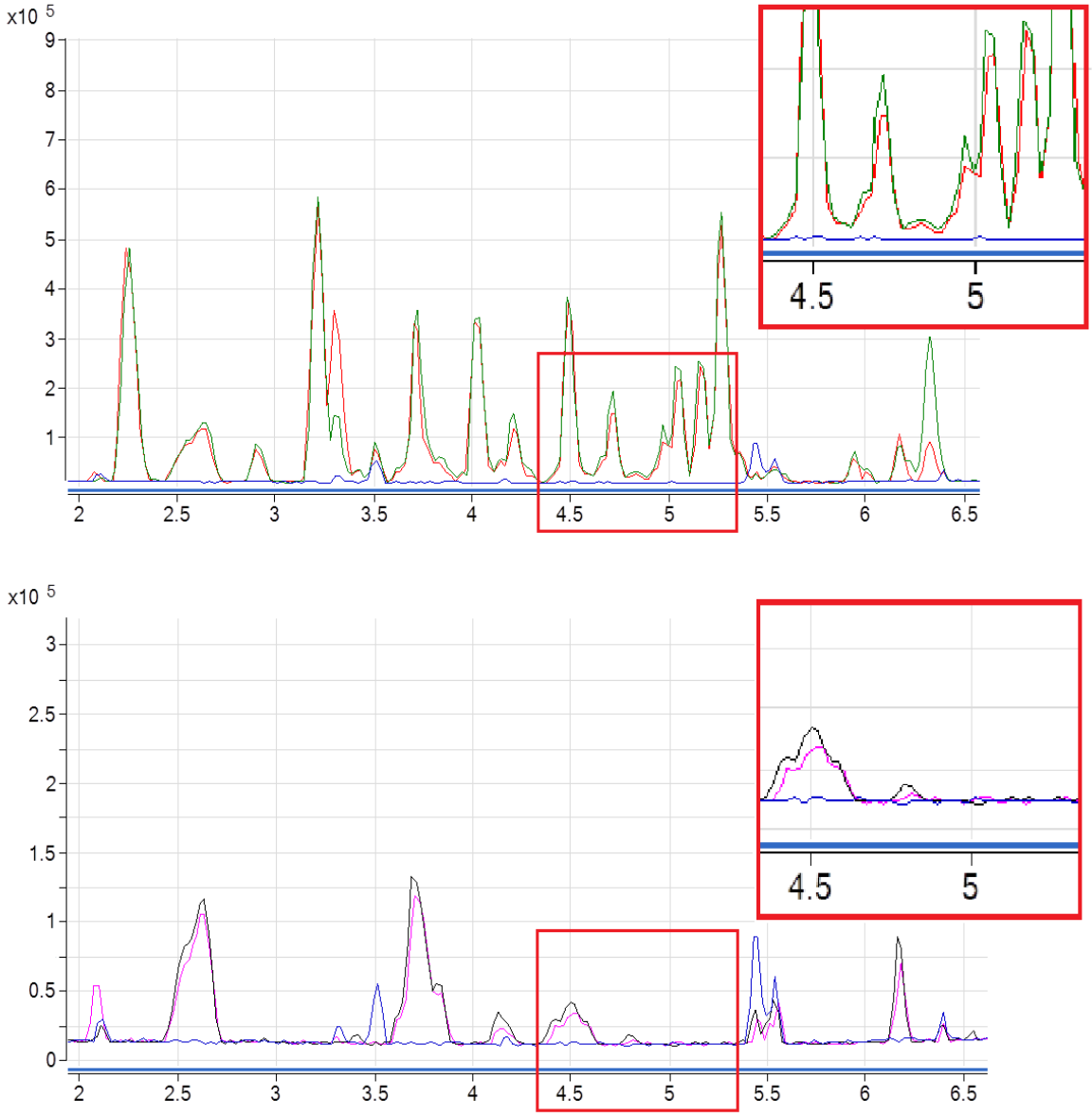


Figure 13**:** Chromatogram (BPC) of (top) the two samples of hair dissolved in the ILC filtered through the Microsep™ Advanced Centrifugal Device, 10 kD mass restricted filters (green and red chromatograms), and (bottom) the two samples of hair dissolved in the ILC and extracted through the usual LLE method (pink and black chromatograms). Both overlayed with the neat standard (neat drug mix reconstituted in 0.1 % formic acid buffer, blue chromatogram)

Table 22: Assessment of mass restricted filters through the comparison of drug recoveries using the standard LLE method, both with and without hair, and using the Microsep™ Advanced Centrifugal 10 kD mass restricted devices. Filter method (A) has the addition of n-BuCl to the filtrate after filtration, whereas filter method (B) has the n-BuCl centrifuged through the filter as wash. Results present as % recovery (±1 SD), where n=2 for the traditional LLE method and n=1 for each of the filtered methods

	No filter/no hair	No filter/with hair	10kD filter (A)	10kD filter (B)
AMP	25.9 ± 7.42	10.8 ± 0.486	11.8	14.4
MDMA	32.1 ± 12.2	21.6 ± 4.79	8.52	13.9
METH	63.4 ± 63.2	29.2 ± 1.79	37.3	34.0
MOR	Not detected	Not detected	Not detected	Not detected
COD	90.1 ± 9.32	83.0 ± 8.38	44.3	63.3
TEM*				
OXAZ*				
NOR*				
DIAZ*				
MET	113 ± 6.22	84.1 ± 3.03	23.2	71.5
COC	79.1 ± 10.7	43.7 ± 4.47	9.85	32.6
OXY	82.4 ± 0.591	39.9 ± 5.06	19.2	40.9

* Analytes not tested as the laboratory did not have permits for these drugs at the time of the experiment.

4.4.4 Drug Stability and Recovery

Drug Stability

Stability of each analyte in the microwave was assessed at 60 °C, 65 °C and 70 °C, using a power output of 60W. At all temperatures, there was a significant amount of degradation (refer to Table 23). Even at the lowest temperature setting of 60 °C, 5 of the 11 drugs able to be detected had a loss of >50 %. With amphetamine, MDMA, methamphetamine, oxazepam and oxycodone having a percentage loss of 86 %, 72 %, 59 %, 66 % and 53 %, respectively. Oxazepam is a particular concern as this analyte could only be detected in one replicate at 65 °C and one replicate at 70 °C, whereby both samples experienced a >95 % loss. This was improved by lowering the temperature setting to 60 °C, but the 66 % loss is still a concern. The recovery of the target analytes from artificially incorporated

hair was assessed using the 60 °C set temperature as the microwave temperature could not be lowered further. This allowed a lower level of degradation than the higher temperatures trialled, and results at this temperature were compared to the current 18-hour methanol extraction at 45 °C.

Table 23: Percent loss (±1 SD) of each target analyte after being microwaved in the ILC at 60, 65 and 70 °C, and extracted using the LLE method (where n=3)

	60 °C	65 °C	70 °C
AMP	86.4 ± 11.7	87.3 ± 8.90	73.2 ± 22.0
MDMA	71.8 ± 11.4	75.7 ± 7.71	59.3 ± 29.1
METH	59.2 ± 23.7	55.3 ± 12.1	not detected
MOR	not detected	not detected	not detected
COD	43.1 ± 8.64	37.3 ± 18.1	76.5*
TEM	48.0 ± 13.7	77.2 ± 15.1	29.7 ± 28.6
OXAZ	66.4 ± 21.0	97.5*	96.3*
NOR	32.8 ± 18.9	53.4 ± 27.8	56.4 ± 0.357
DIAZ	27.6 ± 18.3	36.0 ± 47.7	25.2 ± 14.0
MET	37.0 ± 31.2	27.6 ± 60.0	19.4 ± 1.49
COC	39.3 ± 35.6	90.2 ± 6.03	80.4 ± 5.39
OXY	53.1 ± 33.5	92.6 ± 2.34	91.3 ± 6.80

*Analyte only detected in one ILC digested sample

Recovery and comparison to current method

The recovery study assessed how effectively the ILC digestion method coupled with the LLE method could extract the target analytes from hair that has been artificially incorporated with drugs (refer to Chapter 2.2 for drug incorporation method). The samples analysed in this section of the project were not only 'dirty' (i.e. had a number of interfering peaks), but a number of the compounds (refer to Table 24), including codeine, temazepam, oxazepam, nordiazepam and oxycodone, were simply not detected in any of the ILC digested samples. In routine operation in the Forensic Toxicology laboratory where this instrumentation was located, the criteria which must be met for detection and identification of a drug are retention time (within ±0.2 minutes of a standard), M+H

(within ±2 mDa), signal area (>15,000) and signal-to-noise ratio (>3:1). These are consistent with agreed criteria among Australian and New Zealand Forensic Toxicology Laboratories [167]. If all these criteria are met then the presence of an analyte is confirmed. However, for this investigation the signal-to-noise criteria was excluded due to the high level of background noise and interference witnessed in the method development stage. For a drug to be considered 'detected' in this section of the experimental, both the retention time and M+H needed to match criteria. As well as the analytes listed above not being detected in any of the samples, two of the remaining analytes, diazepam and cocaine, could only be detected in one of the ILC digested replicates. This may be due to either the stability or extraction mentioned previously. MDMA was the only analyte that could be detected in every ILC digested sample. However, all compounds were detected in the 18-hour methanol digestion.

Table 24: Recovery (±1 SD) of each target analyte from artificially drug incorporated hair using the ILC microwave digestion at 60°C and LLE method. Results presented as a percent recovery of the samples extracted via the overnight incubation (18 hours, 45 °C) in methanol (where n=3)

	% Recovery (± 1 SD)
AMP	413 ± 377
MDMA	203 ± 366
METH	3770 ± 5340
MOR	Not detected
COD	Not detected
TEM	Not detected
OXAZ	Not detected
NOR	Not detected
DIAZ	53.5*
MET	143 ± 21.5
COC	40.5*
OXY	Not detected

*Only detected in one ILC digested sample

Additionally, if detected in the ILC digested samples the peaks were of very poor quality, i.e. poor peak shape, shifted retention time and/or mass, or high level of noise and interferences. Amphetamine and methamphetamine were able to be detected in most of

the ILC digested samples, but the level of background noise (refer to Figure 14) would usually be considered too much for drug confirmation. If the signal-to-noise ratio criterion was employed, amphetamine, methamphetamine and MDMA would be considered 'not detected' in all of the ILC digested samples. These interferences could also have contributed to the inflated percent recoveries, making these values inaccurate. The 18-hour methanol incubation samples all produced clean peaks with minimal background noise, which fit all the necessary inclusion criteria. Further validation experiments were not conducted due to ILC digestion method being deemed unsuitable for the use in forensic toxicology.

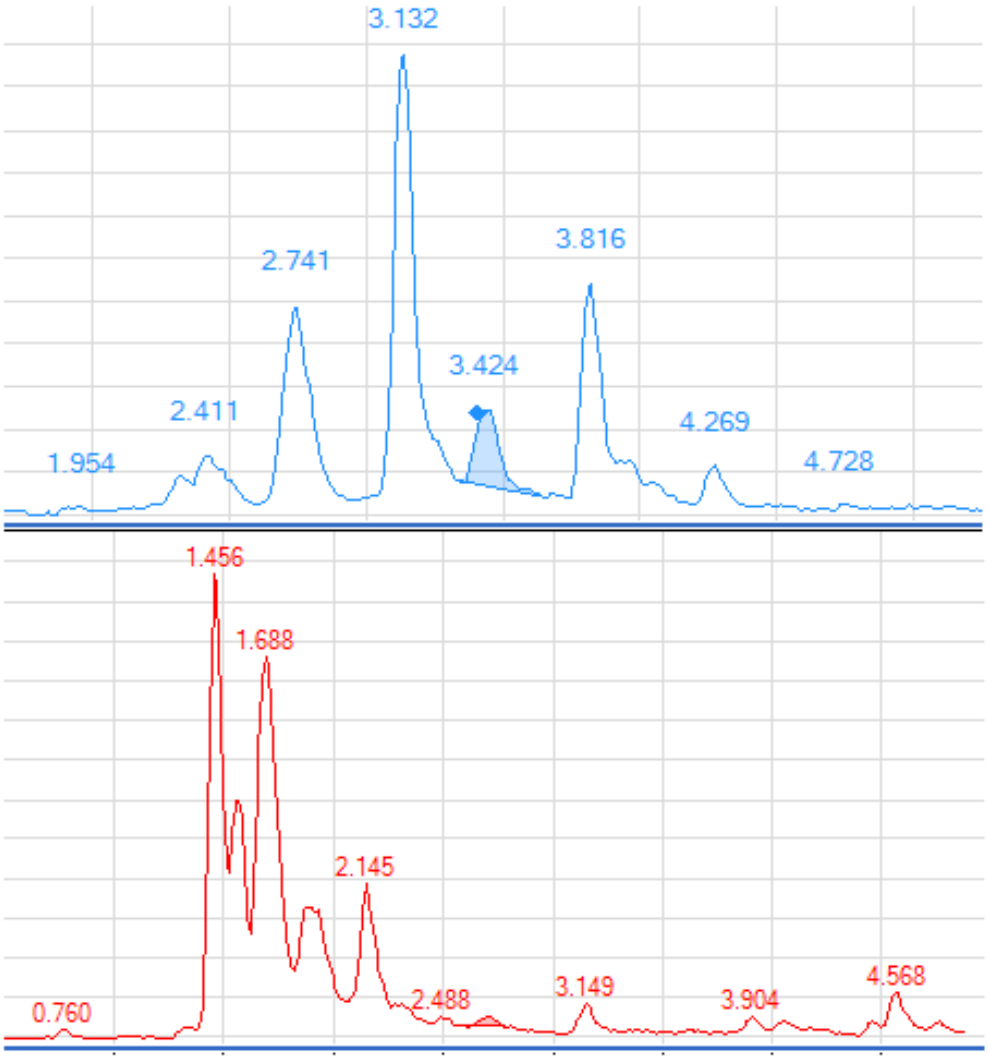


Figure 14: Chromatograms (EIC) of Methamphetamine (top) and amphetamine (bottom) detected in the hair using the ILC digestion and LLE extraction method

4.4.5 Future Work and preliminary studies

Use of ILC for analysis of heavy metals in hair

Due to the instability of the drugs during heating and the difficulty with extraction and clean up, the use for this ILC for dissolution of hair for drug detection may not be suitable in toxicology. However, it may be useful for analysis of thermally stable analytes, such as heavy metals or elemental analysis. The current methods of sample preparation for heavy metal analysis in hair involve either dry ashing, or heated acid digestion. Dissolving the hair in the ILC may offer a safer and more efficient method of sample preparation. This was investigated briefly by digesting 20.8 mg of cleaned hair in 0.5 mL ILC to completion, at 150 °C and analysing through ICP-MS analysis.

The results presented in Table 25 indicate that using the 1 in 1000 dilution there were no detectable traces of the target heavy metal in the blank (hair free) ILC. At these dilution levels it was possible to detect endogenous levels of copper, zinc and lead in the hair. Cadmium was also detected in the hair at low levels at the 1 in 100 dilution. However, at this dilution there were also low levels of zinc, cadmium and lead present in the blank ILC. The difference in cadmium concentration in the hair and blank ILC is too small to discriminate between, and hence the cadmium detected in the hair sample is likely from the ILC not the hair. Matrix effects may be more abundant in the 1 in 100 dilution when compared to 1 in 1000 dilution resulting in the differing calculated concentrations on the metals between the 1 in 100 and 1 in 1000 dilutions. This can be seen by the increase in internal standard recovery (refer to Figure 15) from where the 1 in 100 dilutions started analysis (sample 20). If no matrix effects were present the internal standard recovery would remain at 100 %.

As the hair donor had not been exposed to high levels of the analytes of interest, all detected traces are at an endogenous level. A previous study by Goullé *et al.* [58] used a more traditional sample preparation method by digesting the hair in nitric acid at 70 °C for 1 hour. Goullé *et al.* determined the median endogenous concentrations of copper, zinc and lead in forty-five healthy volunteers to be to be 20.3 ng/mg, 162 ng/mg and 0.41 ng/mg respectively, which is significantly lower than the concentration presented in Table 25. Only one hair sample was used for this investigation, and hence further studies are required to determine if the ILC digestion method is able to extract more of the target

analyte compared to the heated acid digestion methods, and to determine the mean endogenous concentration of the target analytes from multiple sources of hair.

Table 25: Concentration of heavy metals in hair (20.8 mg) from a healthy volunteer and in a blank (hair free) ILC sample determined using ICP-MS analysis after a 1 in 1000 dilution (top) and 1 in 100 dilution (bottom)

	Sample	mass (mg)	Cu ng/mg	Zn ng/mg	As ng/mg	Cd ng/mg	Hg ng/mg	Pb ng/mg
1 in 1000 Dilution	ILC blank	nominal 20 mg	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	ILC + Hair	20.8	200	540	<LOQ	<LOQ	<LOQ	4.2
	LOQ		12.5	12.5	1.25	0.25	0.25	0.25
1 in 100 Dilution	ILC blank	nominal 20 mg	<LOQ	1.61	<LOQ	0.03	<LOQ	0.10
	ILC + Hair	20.8	140	440	<LOQ	0.05	<LOQ	3.2
	LOQ		1.25	1.25	0.125	0.025	0.025	0.025

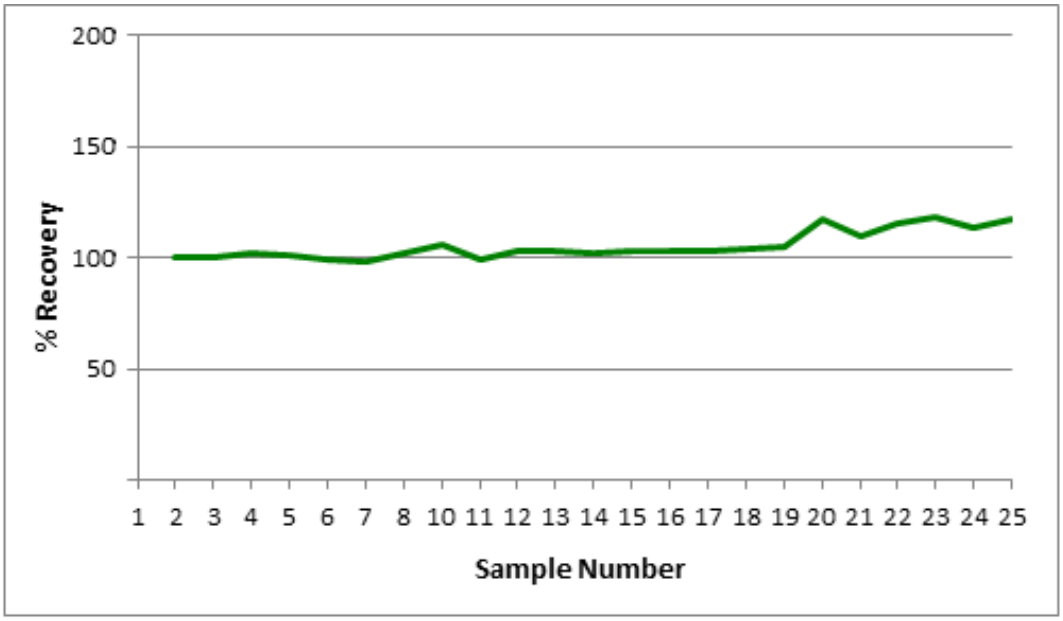


Figure 15: Monitoring of internal standards for quality control for IC-PMS analysis

4.5 Conclusions

This chapter investigated the use of a urea/choline chloride composite at a 2:1 molar ratio in the digestion and extraction of drugs from hair, which currently not been investigated in literature. Using this particular ILC, achieving a sufficient level of dissolution is difficult at temperatures low enough to avoid potential drug degradation. Conventional heating methods require either high temperatures (~150 °C) or long periods of time to disassemble the hair. Higher temperatures cannot be used for the purpose of extracting drugs as it will cause degradations, and lower temperatures (between room temperature and 50 °C) requires overnight mixing and still only achieves a small amount of dissolution, i.e. removal of cuticle scales. This level of dissolution may be enough to allow for extraction of drugs, however the current method already consists of an overnight incubation so would not save any time. The added clean-up step (LLE) increases the overall time needed for sample preparation even more. With the assistance of microwave digestion, the dissolution process takes less time, but the temperature could not be reduced. Using lower set temperatures and lower power outputs can reduce the temperature reached by a few degrees, but very little dissolution occurs. It is possible that using the lower set temperatures and lower power outputs and extending the microwaving time could result in higher dissolution rates, but the temperatures were reaching above 80 °C even when set to cool immediately. No matter what dissolution conditions were used, extraction and clean-up was also difficult due to the high viscosity of the ILC, resulting in low recoveries. While this technique for drug analysis in hair was unsuccessful, this is the first time an ILC has been used for this purpose. Additionally, the short but promising preliminary study on detection of heavy metals in hair using the ILC, shows that this method may still be usable in the hair analysis industry, just not for drugs. This is also new work that has not yet been investigated or published in the literature.

Chapter 5

The effect of Olaplex® and bleach treatments on drug detection and the overall structure of hair

This chapter investigates the effect of the hair treatment product Olaplex® on i) hair microstructure and ii) the detectable concentrations of drug in the hair. The results discussed include:

- *How the Olaplex® treatment, along with a bleach treatment effect the detectable drug concentration in hair*
- *Investigation into whether Olaplex® treatment can be detected in hair case samples where treatment is suspected using techniques available to forensic laboratories (SEM and ATR-FTIR)*

5.1 Introduction

Although hair is thought to be very stable forensically, it is well known that constant exposure to natural factors, e.g. sunlight, weather, water, pollution, etc. can damage hair. This damage can also have a detrimental effect on measured drug concentrations within the hair. Similarly the use of cosmetic hair treatments, such as bleaching, colouring and perming, and heat treatments including straightening, styling and blow drying, can cause damage to the keratin structure of the hair and can significantly influence drug concentration measurements. A number of studies have shown a drastic decline in analyte concentrations after cosmetic treatments, with the most severe decreases seen in bleaching treatments [91-93, 98, 168-174]. Previous studies have indicated that this decrease is a result of a combination of degradation to the drug itself and degradation to the hair resulting in the release of drug. Yegles *et al.* [174] found that the peroxide treatment caused a reduction in the concentrations of a number of benzodiazepines as well as codeine, 6-monoacetylmorphine and morphine. However, they did not report any degradation products. Kerekes *et al.* [175] found a reduction in ethyl glucuronide (EtG) content in hair after bleaching is the result of chemical degradation of the EtG and a leaching out effect from the hair . Other studies have verified the formation of cocaine oxidation products, including hydroxynorcocaine and dihydroxycocaine, as well as the formation of ecgonine methylester and benzoylecgonine in the presence of hydrogen peroxide [77, 176]. These reaction products are more hydrophilic resulting in easier removal from the hair during washing. Cypers *et al.* [77] has also indicated that cocaine and its reaction products are being removed from the hair during the bleaching and the wash following the treatment.

The use of cosmetic hair treatments such as bleaching, colouring and perming, and heat treatments causes damage to the keratin structure of the hair. The keratin molecules that make up the hair contains a number of amino acids, one of which being cysteine (refer to Figure 16). Cysteine units provide an abundance of thiol groups (-SH) along the chain which often form disulphide bonds (-S-S-) with other thiol groups within the chain, or with other cysteine units to give cystine, giving the hair curls, buoyancy and strength [1, 3, 7].

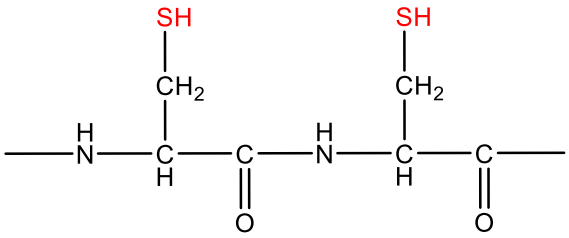


Figure 16: General structure of two cysteine residues forming a keratin fibre of hair

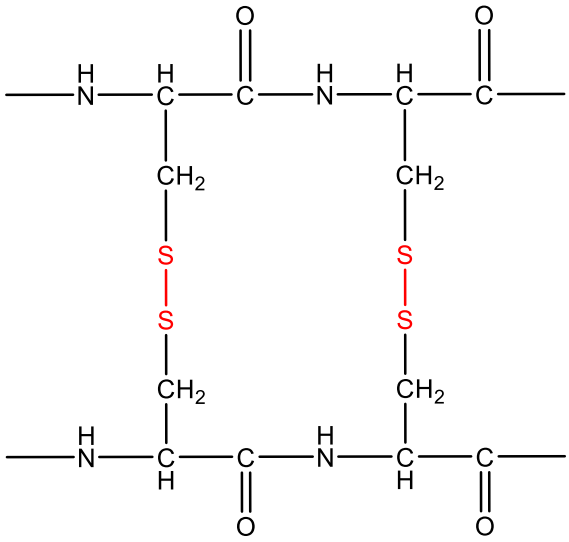


Figure 17: General structure of a cysteine chain bonded to another cysteine chain through a crosslinking disulphide bridge, present in the keratin fibres of hair

As the hair becomes damaged by environmental and chemical factors, these disulphide bonds can break, reverting back to a free thiol groups. Even repeated washing with slightly alkaline shampoo (pH 8.5) damages the hair by breaking more and more of the disulphide bonds [99]. These breakages result in the hair becoming frizzy and brittle, increases the level of static in the hair resulting in “fly aways” and reduces the shiny appearance as frizzy hair catches light unevenly making it appear dull.

The Olaplex® treatment is marketed as a bond multiplier, which claims to repair and rebuild broken crosslinking disulphide bonds [99, 100]. This is accomplished when the main constituent in Olaplex®, bis-aminopropyl diglycol dimaleate (refer to Figure 18), undergoes a nucleophilic addition reaction (Michael addition) resulting in a new bridge (refer to Figure 19).

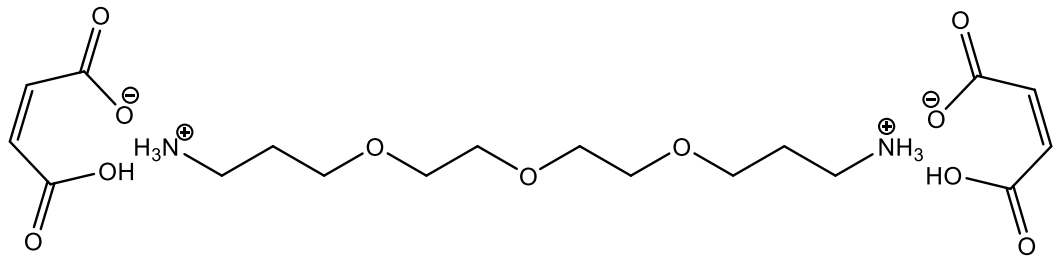


Figure 18: Bis-aminopropyl diglycol dimaleate structure

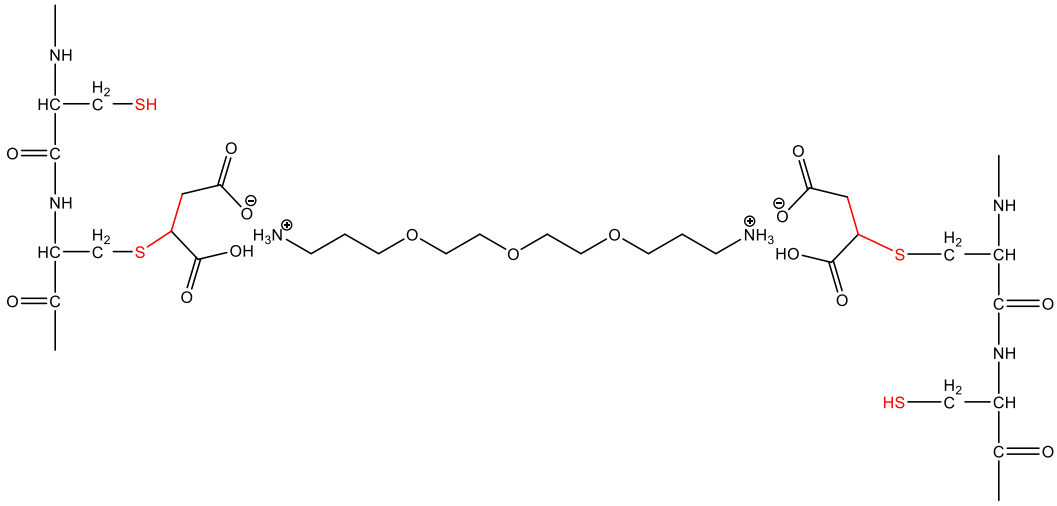


Figure 19: Structure of the new bridge between cysteine chains in hair after using the Olaplex® binding formation

When used to treat hair, the Olaplex® treatment is used in 3 stages: Olaplex® No. 1 Bond Multiplier, Olaplex® No. 2 Bond Perfector and Olaplex® No. 3 Hair Perfector. Olaplex® No. 1 Bond Multiplier, a liquid, is either mixed in with the hair colour (usually a beach powder and peroxide mix) to reduce the extent of damage done to the hair during the colouring process, or applied to virgin hair without colour or additional treatments as a repair treatment. Olaplex® No. 2 Bond Perfector, a conditioning cream, immediately follows the No. 1 treatment and is left on the hair for approximately 20 minutes before being rinsed off, and the hair shampooed and conditioned. Both Olaplex® No. 1 and No. 2 are in-salon treatments, and are applied to the hair by haircare professionals. Pressley and Hawker, the inventors and patentees of the Olaplex® treatment, claim that the thiols will remain bound for at least a week, and hence the Olaplex® No. 3 Hair Perfector, a conditioning cream, is a take home treatment to be used once a week prior to regular shampoo and conditioning [99].

This binding agent can also be used for perming treatments as well as repair to damaged hair. Traditional perming methods use a reducing agent (e.g. ammonium thioglycolate and or dithiothreitol) to break disulphide bonds apart, and then neutralize with hydrogen peroxide. The binding formation (Olaplex®) can be used instead of the hydrogen peroxide. Pressley and Hawker claim that by using the Olaplex® binding formation instead of the hydrogen peroxide neutralisation, the hair retained similar level of curl after 40 washing and drying cycles, but resulted in the hair having more sheen and less frizz in comparison to using the hydrogen peroxide [99].

With the increasing number of people using this treatment, both in-salon and at-home, it is important to understand the potential effect it has on the drug concentration in hair. Currently, there is no literature on how Olaplex® can i) affect drug concentrations in hair, ii) interfere with toxicological analysis, iii) affect the structure of hair on a microscopic level, or iv) whether Olaplex® treatment can be detected in hair case samples where treatment is suspected. This chapter investigates all of these points.

This chapter looks into the concentration of various drugs in hair prior to treatment and after each stage of the Olaplex® treatment alone and in conjunction with a bleach treatment. The overall damage and repair to the structure of hair through the bleaching and Olaplex® treatments was also assessed through SEM analysis of treated hairs. ATR-FTIR was also used to determine if Olaplex® could be detected in hair samples following treatment

5.2 Experimental

5.2.1 General

The original Olaplex® brand treatments No. 1, 2 and 3 were used to treat hair. Hair being bleached was treated with Results brand bleach powder and hydrogen peroxide cream (20 vol., 6 %). Garnier® Fructis fortifying shampoo and conditioner for normal hair was chosen as the standard weekly wash as it is widely available in a number of countries and regions.

5.2.2 Hair sample collection and preparation

A lock of drug free hair (hair sample 4, approximately 7 g, 15 cm in length – refer to Table 4 for more details) was collected from a healthy volunteer and washed with methanol (2x 100 mL) and spiked with analyte based on a method described by Martin *et al.* [133] (refer to Chapter 2.2). The lock of hair was soaked in the DMIX HAIR solution (200 mL) for 7 days under nitrogen atmosphere and away from light. The DMIX HAIR solution was then removed, and the spiked hair was washed extensively to ensure any remaining analyte was completely incorporated into the hair. As the hair was treated as a large lock, rather than finely cut pieces, a rigorous washing procedure was undertaken than in previous chapters. Briefly, the hair was washed by swirling or sonication in methanol (100 mL) for 1 minute and discarding the solvent (25 times). This was followed by swirling in MeOH:H_2O (1:1 v/v, 100 mL) for 1 min, 5 times, and finally swirling in water for 1 minute, 5 times. The hair was left to dry and a 20 mg sample from the bulk lock was collected and washed with methanol (2mL, 30 second), and extracted and analysed. The solvent from the wash was also collected and analysed to ensure there was no drug leaching from the hair, and the concentration of drug inside the hair was high enough to continue with the experiment. The bulk lock of hair was sectioned off into 4 separate locks for treatment (refer to Table 26).

For the samples which underwent the full Olaplex® treatment, but were not bleached (samples 2 and 4, refer to Table 26), the Olaplex® No. 1 (7.5 mL) was diluted in water (42.5 mL) and added to the dry locks of hair until saturated and left to stand at room temperature for 5 minutes. Without rinsing, the Olaplex® No. 2 was massaged into the locks and left to stand at room temperature for a further 20 minutes. The amount of Olaplex® No. 2 used was enough to sufficiently cover the entire lock of hair. The hair was

then rinsed with water, shampooed and conditioned, rinsed again and left to dry at room temperature.

For the sample that was bleached and treated with the Olaplex® (sample 6), bleach powder (35 g) was mixed with peroxide cream (70 mL) until it formed a thick cream consistency. Immediately after the bleach and peroxide were mixed, Olaplex® No. 1 (3.75 mL) was also mixed in. A generous amount of the bleach/peroxide/Olaplex® mix was applied to the lock of hair and left to stand at room temperature for 25 minutes. The hair was rinsed to remove all of the bleach treatment, and Olaplex® No. 2 was applied and left to stand at room temperature for a further 20 minutes. The hair was then rinsed with water, shampooed and conditioned, rinsed again and left to dry at room temperature.

For the bleached hair, with no Olaplex® treatment (sample 5), bleach powder (35 g) was mixed with peroxide cream (70 mL) until it formed a thick cream consistency and added to dry hair. This was left to stand at room temperature for 25 minutes, and was then rinsed, shampooed and conditioned, rinsed again and left to dry at room temperature.

Table 26: Sample and treatment types, including blank and controls

Sample #	Sample type	Description
1	Blank	No spike, virgin hair, no Olaplex® treatment**
2	Blank	No spike, virgin hair, full Olaplex® treatment* (spike **after** completion of treatment, refer to Chapter 6)
3	Control	spiked, virgin hair, no Olaplex® treatment
4	Treatment	spiked, virgin hair, full Olaplex® treatment
5	Treatment	Spiked, bleached, no Olaplex® treatment
6	Treatment	Spiked, bleached, full Olaplex® treatment

*Full Olaplex® treatment refers to hair being treated with all three stages of Olaplex® (including the in-salon treatments, Olaplex® No. 1 Bond Multiplier and Olaplex® No. 2 Bond Perfector, and the weekly at-home treatment, Olaplex® No. 3 Hair Perfector)

** All samples that did not undergo Olaplex® treatment; regular shampooing and conditioning where Olaplex® No. 3 would usually be used, unless otherwise stated.

The Olaplex® No. 3 treatment was conducted weekly, whereby the treatment was applied to damp (towel dried) hair and left for 10 minutes, then rinsed, shampooed and conditioned and left to dry at room temperature. The remaining hair locks were shampooed and conditioned and left to dry at room temperature, weekly. All dried hair samples were wrapped in foil and stored away from light and heat until the next treatment.

A 1cm segment was collected from the distal end of each hair lock after each treatment step (refer to Table 27). The Olaplex® No. 3 treatment and shampooing/conditioning was performed once per week, but samples were only collected after the first week, 5 weeks and 9 weeks.

Table 27: Collection points for when 1 cm segments were collected from each sample

Collection number	Collection point	Applicable samples
1	Before treatments	All
2	After in-salon treatments, i.e. bleach, Olaplex® No. 1 and Olaplex® No. 2	3-6
3	Olaplex® No. 3 and/or regular shampooing (week 1)	All
4	Olaplex® No. 3 and/or regular shampooing (week 5)	All
5	Olaplex® No. 3 and/or regular shampooing (week 9)	All

5.2.3 Effect of treatment on drug concentration

After the ninth week of Olaplex® No. 3 and shampooing/conditioning treatments, the 1 cm locks from all samples at collection points were extracted, in triplicate, using the 18-hour incubation method (refer to Chapter 2.3) and analysed using the LC-QTRAP® 4000 (refer to Chapter 2.4.2) to determine concentration of each analyte in the hair samples. All samples were cut into 1-5 mm fragments, weighed out into 20 mg portions and washed with 2 mL methanol for 30 seconds prior to extraction. The wash from first of the replicates for each sample was also collected and analysed.

It should be noted that a full statistical analysis was not performed for this investigation due to the complexity of hair samples and the high level of variability between each sample. Additionally, the long sample preparation time and limited instrument availability made it difficult to produce enough samples to make the data statistically relevant.

5.2.4 Assessment of structural damage and repair

Locks of hair that had not been artificially incorporated with drugs were treated with the Olaplex®, bleach+ Olaplex® and bleach treatments mentioned in section 5.2.2 Hair sample collection and preparation. 1 cm segments were collected prior to treatment, after the in-salon treatments and after 5 weeks of Olaplex® No. 3 treatments and/or regular shampooing. From each segment, 6-8 fibres of hair were collected and analysed through SEM analysis (refer to Chapter 2.4.4 for details). Only a small number of images for each sample were captured, but all 6-8 fibres for each sample were assessed along the length of the hair shaft (approximately 0.5-1 cm in length) to ensure the images capture were an accurate representation of the overall analysis. Un-spiked hairs were used in this instance as the artificial incorporation method can cause damage to hair (refer to Chapter 6.3.2, pg. 125), and hence it would be difficult to determine whether any damage was due to the cosmetic treatments or from the drug incorporation.

5.2.5 Assessment of structural damage and repair - single hair assessment

Three strands of hair (untreated) were individually mounted for SEM analysis by wrapping the hair around a piece of silicon wafer (1 cm in length) and secured on the underside (the rough side) with a piece of carbon tape. Using the exposed piece of carbon tape that secured the hair, the silicon wafer was mounted on to a SEM stub. Each of the 3 individual strands of hair was analysed through SEM using the Inspect FEI F50 SEM. This analysis used a 2 kV ion beam and a spot size of 5, and the hairs were not coated prior to analysis. These individual hairs with then treated with either a) the Olaplex® in-salon treatments (No. 1 and No. 2); b) bleaching treatment; and c) bleaching treatment with Olaplex® incorporated into the bleach. Following treatment, the hairs were then imaged again using the same SEM conditions.

5.2.6 Detection of hair treatment using FT-IR and ATR

Strands of hair that had been treated with a) the Olaplex® in-salon treatments (No. 1 and No. 2); b) bleaching treatment; c) bleaching treatment with Olaplex® incorporated into the bleach; and d) untreated, were collected and analysed using Fourier-transform infrared spectroscopy (FT-IR). Attenuated total reflection (ATR) was also used to assessed untreated and bleach treated hair. All of the hair samples presented in this study are after the in-salon stage of treatments. Refer to Chapter 2.4.4 for details on FT-IR and ATR analysis and sample preparation.

5.3 Results and discussion

5.3.1 Effect of treatment on drug concentration

By monitoring the reduction in drug concentrations in the hair throughout the various hair treatments (refer to Figure 20-Figure 23 for graphs), it was found that normal shampoo and conditioning had the least effect on drug concentration (refer to Table 33 in Appendix for concentration values). Most of the analytes exhibited a gradual decrease over the treatment period with the greatest decrease in concentration occurring for methamphetamine with a 50 % loss by the final at-home wash. Oxazepam and methadone were the least effected with no loss in drug over the 9 washes. This supports previous studies that have shown little to no loss of these two drugs as a result of regular washing [97, 98].

Through treating the hair with Olaplex® No. 1 and No. 2, without bleaching (Figure 20-Figure 23 and Table 34 in Appendix) there was a decrease in concentration for 7 of the 12 drugs. Amphetamine, MDMA, methamphetamine, morphine, codeine, cocaine and oxycodone all decreased in concentration by 30-55 % after just the in-salon treatment. Oxazepam, nordiazepam and methadone were the least effected with no observable loss, and diazepam only decreasing in concentration by 12 % after the in-salon treatment. This loss of drug was far greater than observed through standard shampoo and conditioning, which may be a result of the Olaplex® washing the drugs out of the hair during the treatment. Alternatively, this decrease may be due to the Olaplex® locking the drug into the hair making it harder to extract out. During the at-home treatments most of the analytes followed a similar trend to the standard shampooing and conditioning in which there was a gradual decrease in the concentrations, however by the final wash the concentration of diazepam had dropped to below the limit of quantification (LOQ) (25 pg/mg).

Bleaching had the greatest effect on the drug concentration (Figure 20-Figure 23 and Table 35 in Appendix), resulting in a large decrease after just the in-salon treatment. Morphine, codeine and diazepam, with initial concentrations of 232, 293 and 50.3 pg/mg respectively, all dropped to below the LOQ (<12.3, <11.9 and <25.0 pg/mg, respectively) after the bleaching step. By the final at-home shampoo and conditioner treatment, morphine, codeine and oxycodone could not be detected at all in the hair, and MDMA,

methamphetamine, methadone and cocaine had also dropped to below the LOQ (<12.5, <12.5, <11.4 and <12.5 pg/mg, respectively). Amphetamine and nordiazepam, even though they are within the quantifiable range, were both very close to the lower limit, with the lower limits being 12.5 pg/mg and 23.4 pg/mg. It is clear that the bleaching treatment causes a drastic decline in the concentration of drugs within the hair, however, it is not clear how much of this decrease is due to degradation of the target analytes, and how much of it is due to the bleach damaging the hair and allowing target analytes to be washed out of the hair, as previous studies have determined that both contribute to the loss [175]. Additional studies are required to assess the effect on the target analytes and whether any oxidation products are formed through a reaction with the hydrogen peroxide, and how much is being washed out.

The Olaplex® treatment incorporated with the bleach had a similar overall effect to the standard bleaching alone, where there was a large decrease in the detected drug concentration (Figure 20 - Figure 23 and Table 36 in Appendix). However, only Morphine, with an initial concentration of 257 pg/mg, dropped to below the LOQ (<12.3 pg/mg) after the in-salon step. By the final at-home wash all the compounds were still able to be detected, but methamphetamine, codeine, nordiazepam, diazepam, cocaine and oxycodone had also dropped to below LOQ (below 12.5, 11.9, 23.4, 25.0, 12.5 and 10.0 pg/mg, respectively). Amphetamine and MDMA were very close to the lower limit of 12.5 pg/mg with each analyte having a detected concentration of 15.0 pg/mg and 14.0 pg/mg by the final at-home treatment. It is likely that if the hair underwent any additional wash cycles, these two analytes could also drop below the LOQ.

Temazepam (refer to Figure 21) shows some anomalous results, in that the concentration increased over the course of the treatments. Some of the analytes show a sudden increase or decrease at one of the sampling points, but this is most likely due to the inhomogeneous nature of hair, whereas the increase in the temazepam concentration seem to be more systematic. Like all the other analytes there is a large decrease in temazepam concentration after the bleaching step, but then begins to increase with the number of at-home treatments. Previous studies by Ettlinger *et al.* [92, 93] on the effect of thermal hair straightening on drug concentration also detected an increase in parent drug concentrations after treatment. The authors claimed this may be due denaturation of the hair matrix by thermal treatment possibly causing a better extraction of the drug.

It is possible the damage done to the hair through bleaching is causing better extraction. However, the increase in temazepam is also seen in the hairs treated with the Olaplex® treatment without the bleach and the regular weekly shampooing, to an even greater extent. The explanation that damage to the hair is resulting in better extraction is not feasible in this case, and hence further investigation into why this increase is occurring is needed.

Prior to analysis all samples and replicates were washed with 2 mL methanol for 30 seconds, and the wash from one replicate of each sample was collected and analysed (refer to Table 39 and Table 40 in Appendix for results). Amphetamine was present in all washes from the spiked samples, even after 5 at-home treatments, and in some cases after 9 at-home treatments. MDMA and methamphetamine were also present in a number of the washes, but at much lower concentrations and were mostly detected below the LOQ except for the sample collected before cosmetic treatment. These results indicate that even after extensive washing of the hair after the artificial drug incorporation step, some amphetamine, methamphetamine and MDMA still remained on the outside of the hair. Additionally, amphetamine was detected in the wash of the blank (un-spiked) hair and amphetamine and methamphetamine were detected in the blank (un-spiked) hair, in quantifiable amounts even through previous screens have determined this hair sample to be drug free. This has been a common occurrence throughout this project, and is consistent with results presented in the Microwave-Assisted Extraction chapter (Chapter 3.3.4), where amphetamine, methamphetamine and MDMA were all observed to be problematic contaminants with a high degree of crossover from other experiments.

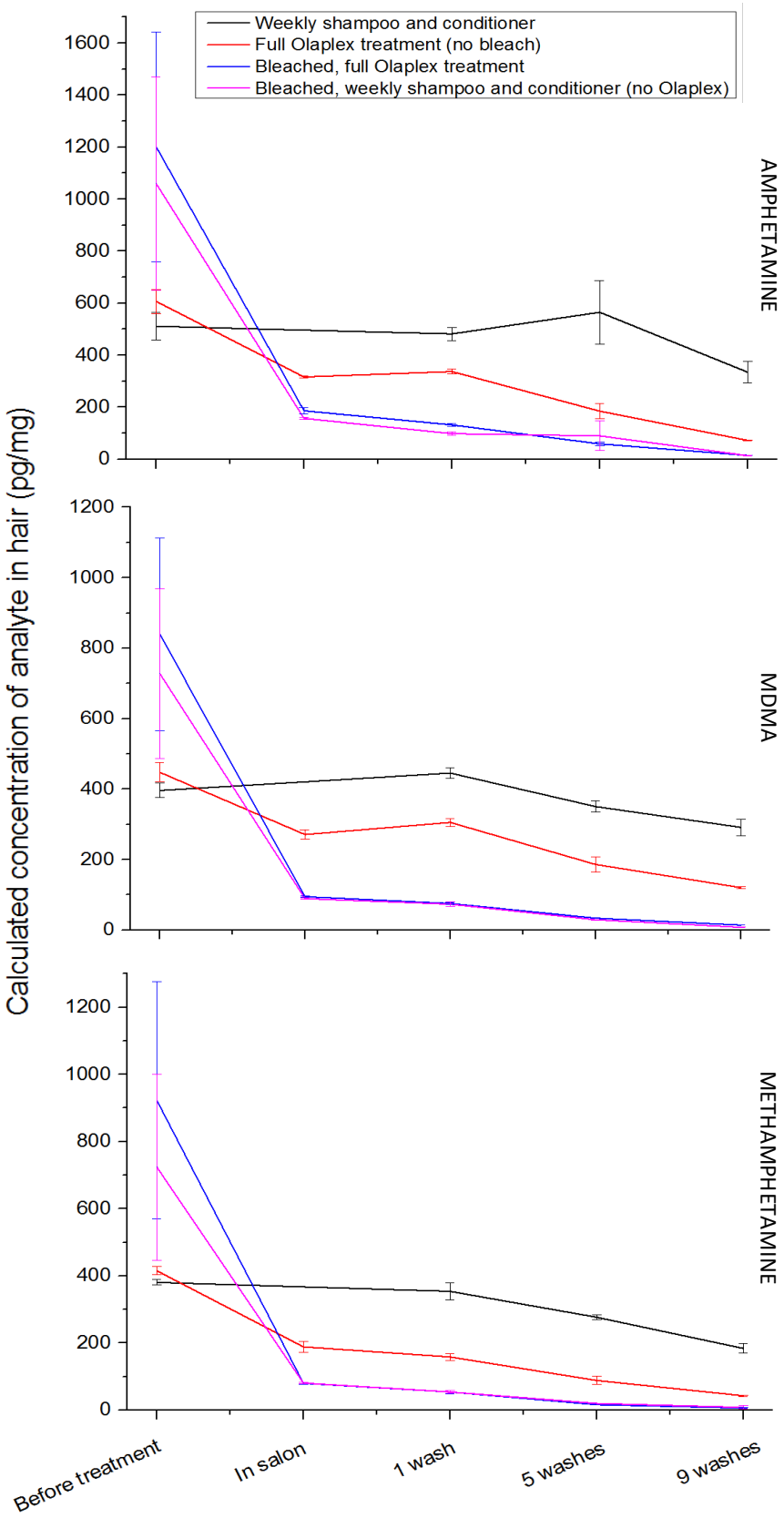


Figure 20: Calculated concentrations (pg/mg ± 1 SD) of after each Olaplex® treatment and washing for (top to bottom) amphetamine, MDMA and methamphetamine (where n=3)

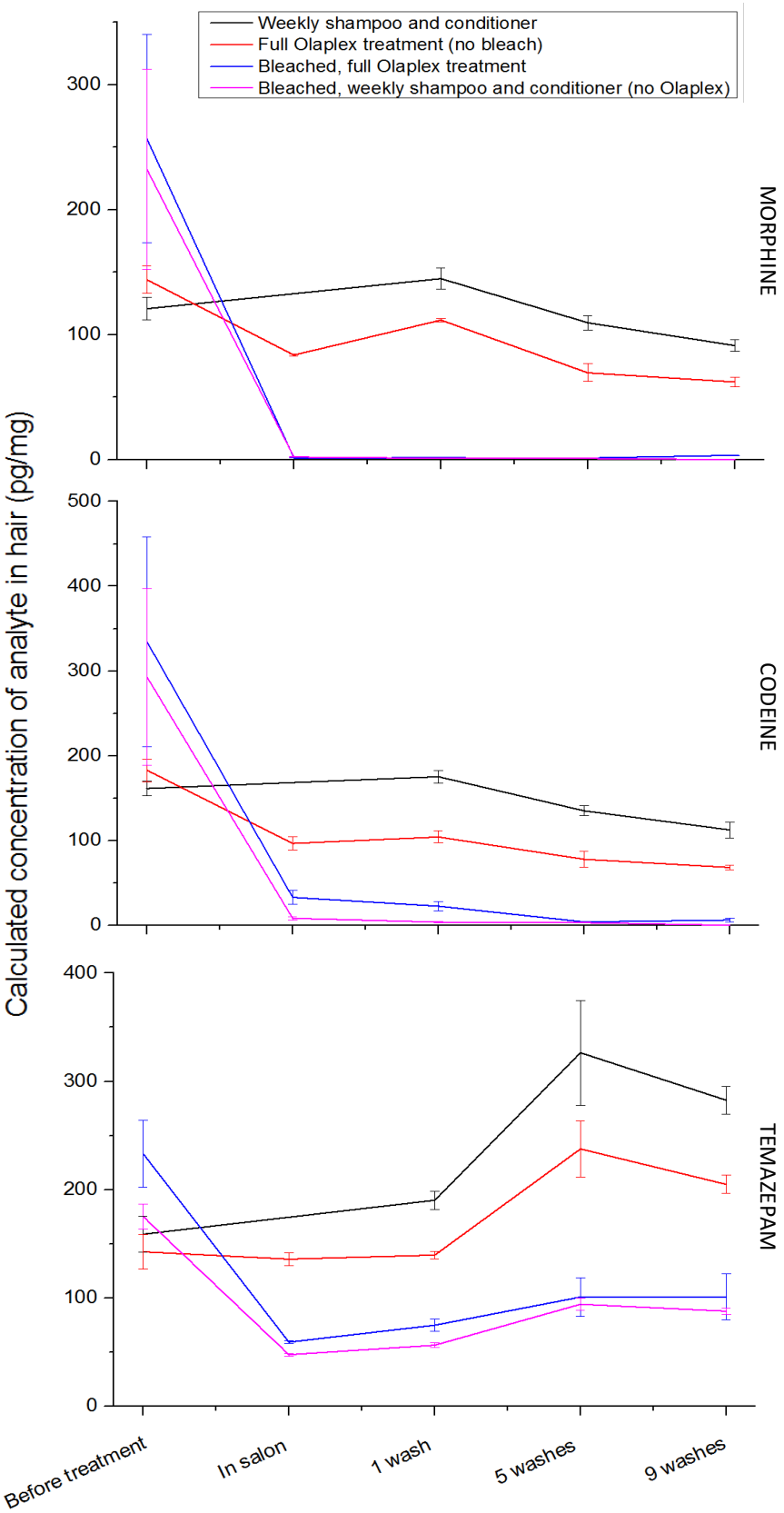


Figure 21: Calculated concentrations (pg/mg ± 1 SD) of after each Olaplex® treatment and washing for (top to bottom) morphine, codeine and temazepam (where n=3)

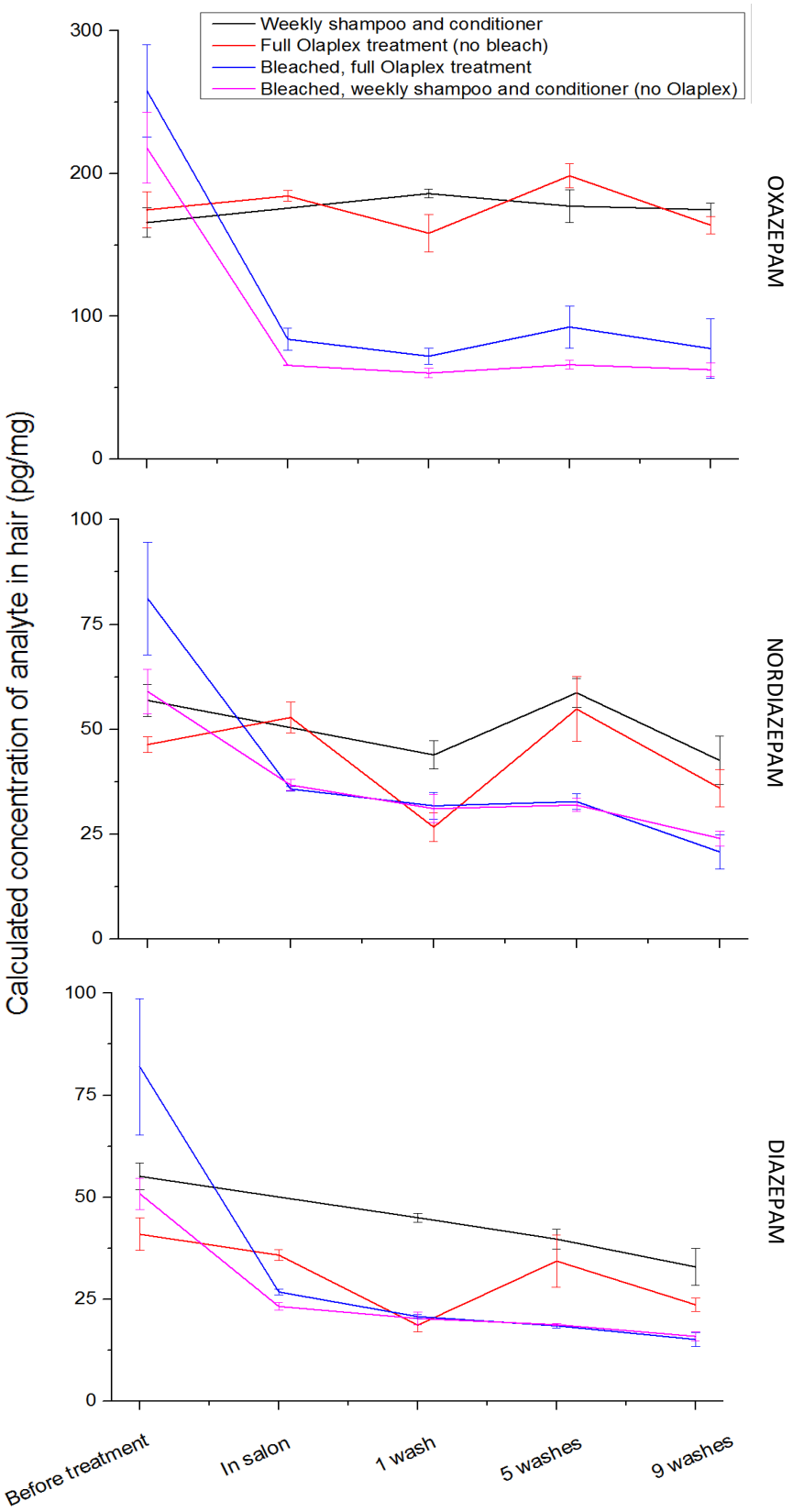


Figure 22: Calculated concentrations (pg/mg ± 1 SD) of after each Olaplex® treatment and washing for (top to bottom) oxazepam, nordiazepam and diazepam (where n=3)

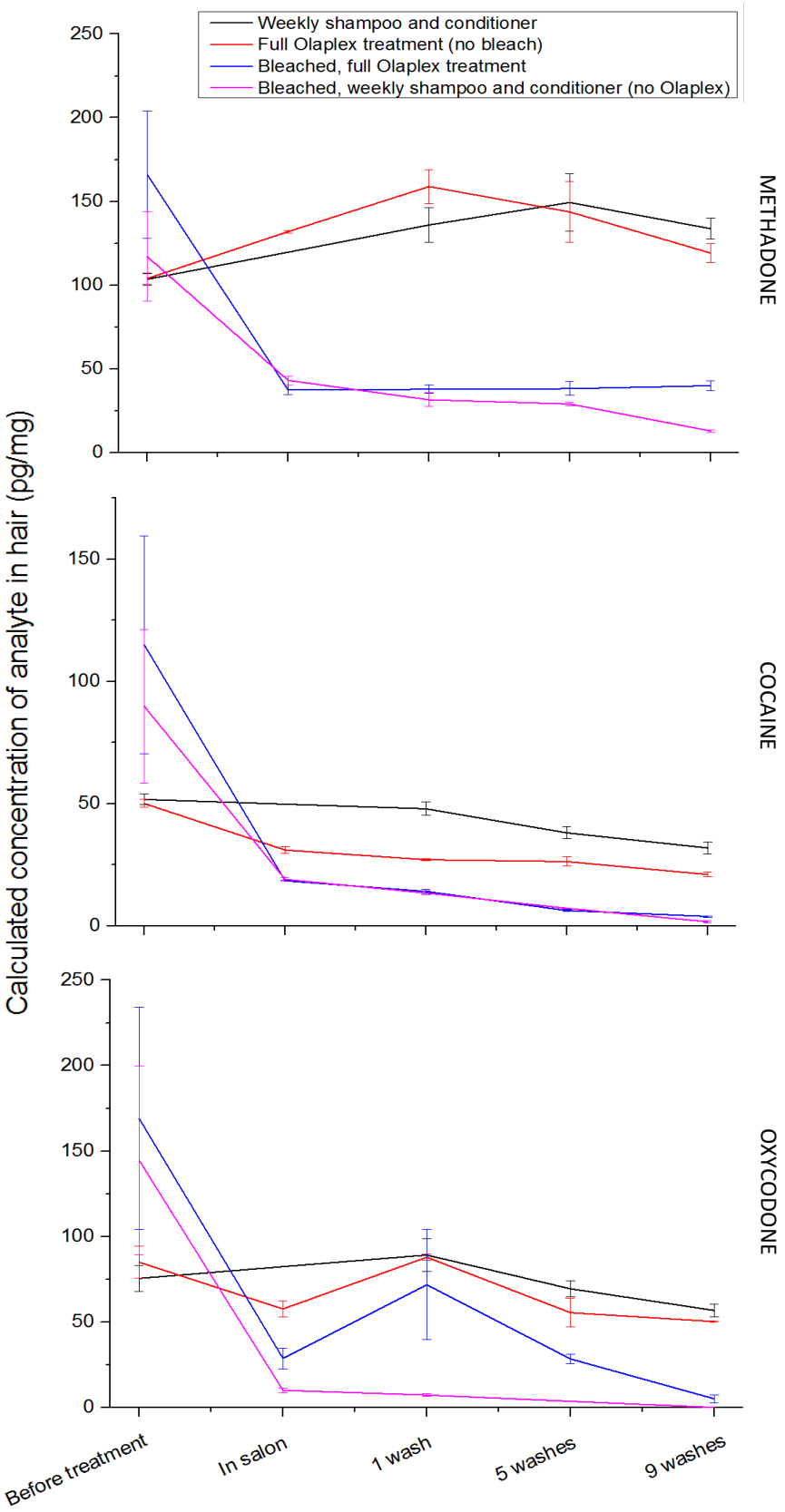


Figure 23: Calculated concentrations (pg/mg ± 1 SD) of after each Olaplex® treatment and washing for (top to bottom) methadone, cocaine and oxycodone (where n=3)

5.3.2 Assessment of structural damage and repair

Through microscopic anaylsis of the hair before and after being treated with bleach and Olaplex® treatments, it was determined that there was no observable difference between the untreated hair and the hair that has been treated with just the Olaplex® treatments (refer to Figure 24). However, the hair used was healthy hair which has had no prior chemical treatments and very little heat treatments (including blow drying).

Bleaching caused severe damage, with Figure 25 (bottom) showing the level of damage that occurred for most of the hairs examined. All of the outer cuticle scales have been stripped and the bleach has started to penetrate down into the inner cortex, and begin to pull apart the keratin fibres. This is a similar effect to the damage caused by the ionic liquid composite in Chapter 4 (refer to Figure 11). Figure 25 (top) shows the level at which the least amount of damage occurred. Some of the cuticle scales have been removed, and the damage has not yet penetrated down into the cortex. However, this was the case for only one of the hairs examined. This inconsistency between hairs undergoing the same treatment could be the result of the bleach mixture not being evenly distributed across the lock of hair causing varying levels of damage. It also may be due to the nature of hair being non-homogenous and the level of damage to each specific shaft of hair prior to any treatments is unknown After 5 additional washes with shampoo and conditioner (refer to Figure 26), the bleached hair seems to be in approximately the same condition as after the initial bleaching, with little to no improvement to the hair shaft. Figure 26 (top) is an example of the hair shafts in the best condition, with the most the cuticle scales having been removed and starting to expose the keratin fibers of the cortex in some sections. Again, this was the case for only one of the hairs examined, with the rest of the hairs examined having the appearance of the shaft in Figure 26 (bottom). Similar to what was observed after the in-salon treatment of the bleach, all of the outer cuticle scales have been stripped and the bleach has started to expose and pull apart the keratin fibres.

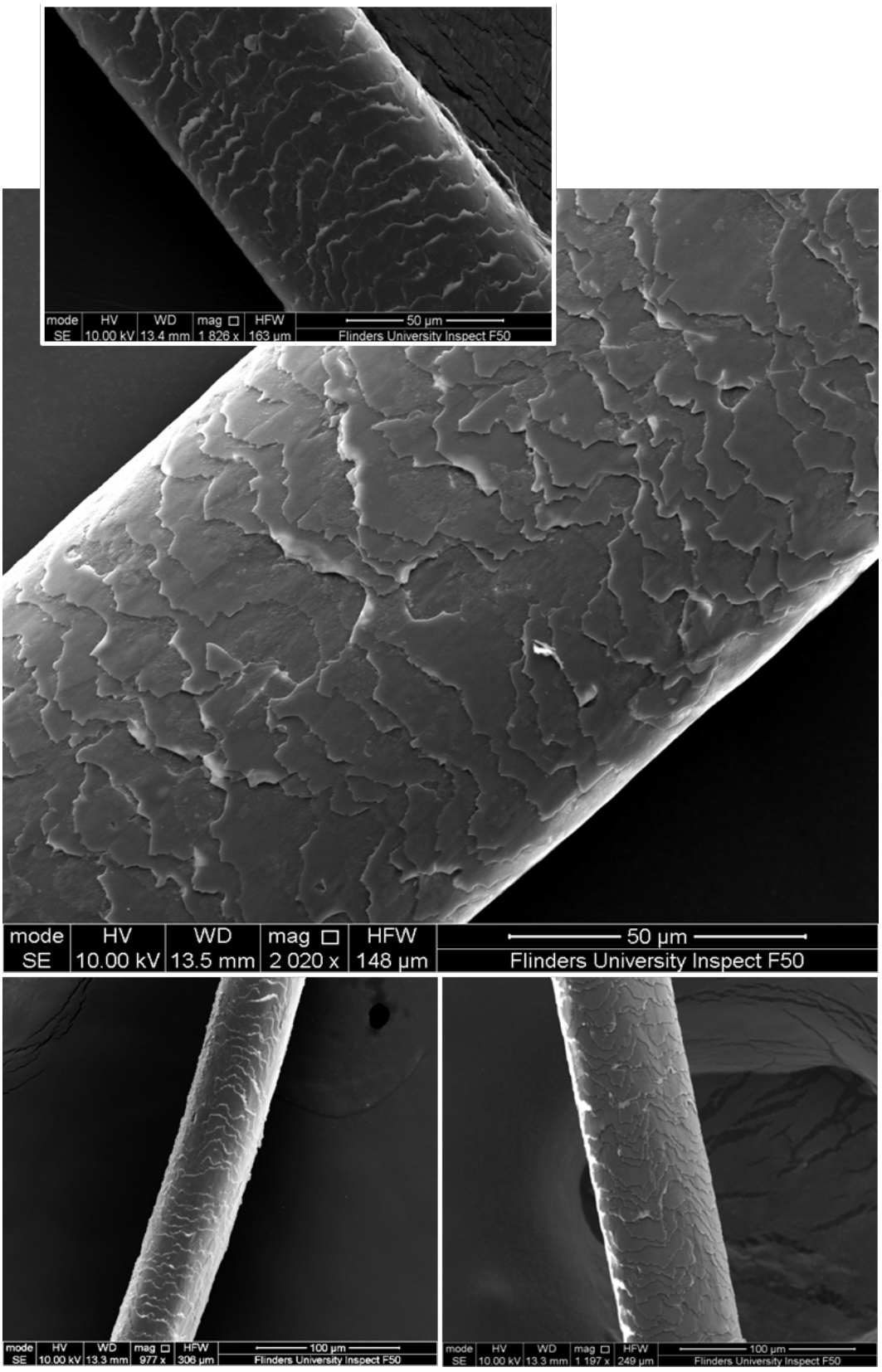


Figure 24: SEM images of healthy, untreated human scalp hair (top), hair treated with the in-salon Olaplex® treatments, No. 1 and No. 2 (middle) and hair treated with in-salon Olaplex® treatment and 5 additional washes using the Olaplex® No.3 take home treatment (bottom left and right). Images taken using Inspect FEI F50 SEM

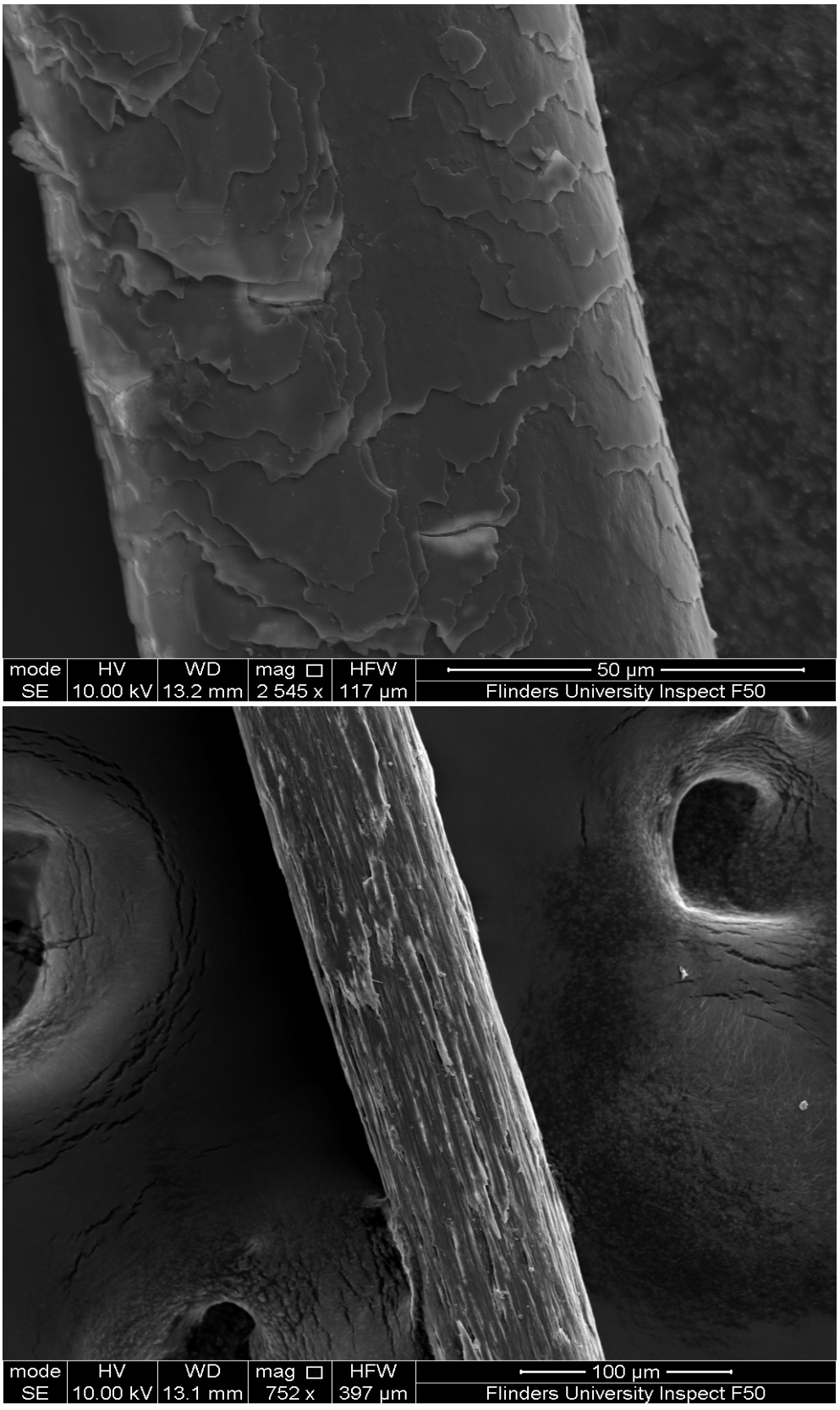


Figure 25: SEM images of human scalp hair that has been bleached, showing the least amount of damage observed (top) and more severe damage cause by the bleach treatment (bottom). Images taken using Inspect FEI F50 SEM

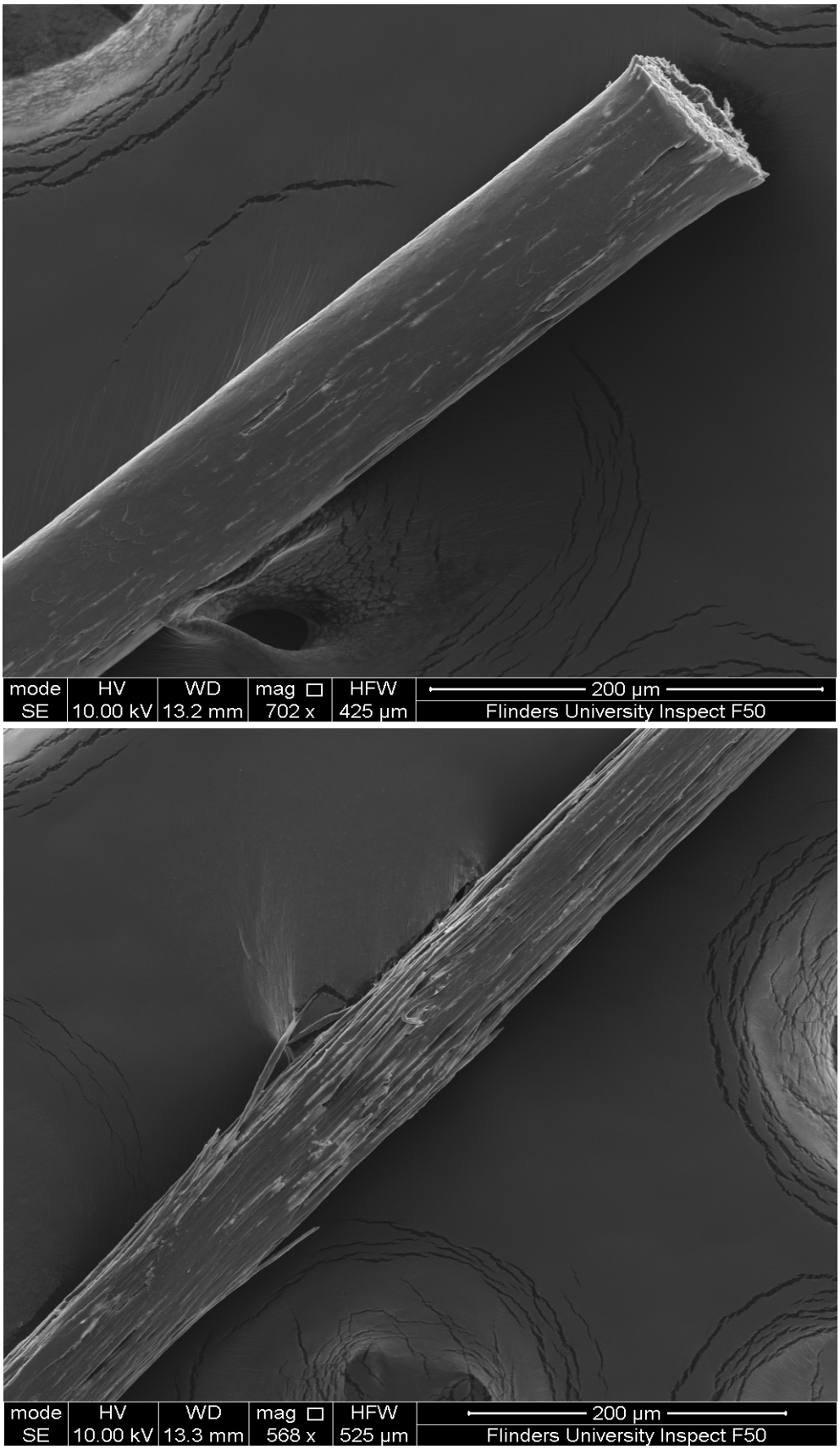


Figure 26: SEM images of human scalp hair that has been bleached and shampoo and conditioned 5 times, showing the least amount of damage observed (top) and the more severe damage observed (bottom). Images taken using Inspect FEI F50 SEM.

Bleaching hair can cause severe damage, but when the Olaplex® No. 1 is incorporated into the bleach treatment followed by the Olaplex® No. 2, the damage is reduced (refer to Figure 27 (top)). After the in-salon treatments the hair shaft was serverely damaged with the inner cortex and the keratin fibres being exposed in some sections. However, the level of exposure was far less than observed after the standard bleaching, and large sections of the cuticle scales are still intact. All the hairs collected and examined after the 5th at-home wash (using Olaplex® No. 3) still had almost all the cuticle scales intact (refer to Figure 27 (bottom)), and the hair appeared to have little to no damage from the bleach. These observations concure with the claims made by Olaplex®, in that the treatment reduces the damage done to the hair during the colouring process, and further deterioration through washing is reduced or halted. The feel of hair and the apprearance to the naked eye is also different beween the Olaplex® treated hair and it's counterparts. The hair used for this study, was healthy wavy hair, with a few curls. After the standard bleaching treatment the hair appreared dull and frizzy, and felt course and wiry when handled. The condition of the hair was improved after five at-home washes. Whereas the bleached hair that was also treated with the Olaplex®, after just the in-salon had far more luster and shine than the standard bleached hair, and after 5 at-home treatments it appeared shinier and formed a ringlet curl after being left to air dry. This curl was much more defined than the waves present in the hair prior to the treatments. The hair that was not bleached, but was also treated with the Olaplex® also developed a more defined curl compared to the un-treated hair (refer to Figure 45 in Appendix for images).

Since the binding agent has been reported to last for approximately 1 week, the Olaplex® No. 3 treatment was performed on a weekly basis, as recommended, to best replicate 'real world' senarios. However, during this study the quantity of Olaplex® used on each sample was far greater than what would usually be used, there were no additional washes, i.e. mid-week shampoo and conditionng in between these treatments, the hair was left to dry at room temperature after each treatment and the hair was stored wrapped in aluminium foil away from light in between the treatments. This does not mimic 'real world' situations, as it is likely the hair is being washed more than once a week, and exposed to potentially damaging elements such as blowdrying the hair rather than being left the air dry, friction and abrasion from brushing and styling the hair, and exposure to natural elements, i.e. sun, wind, rain, etc.. Hence, the conditions in which

these treatments were performed gave the Olaplex® the best possible advantage and may not accurately represent the effect of Olaplex® on the hair in real-life situations.

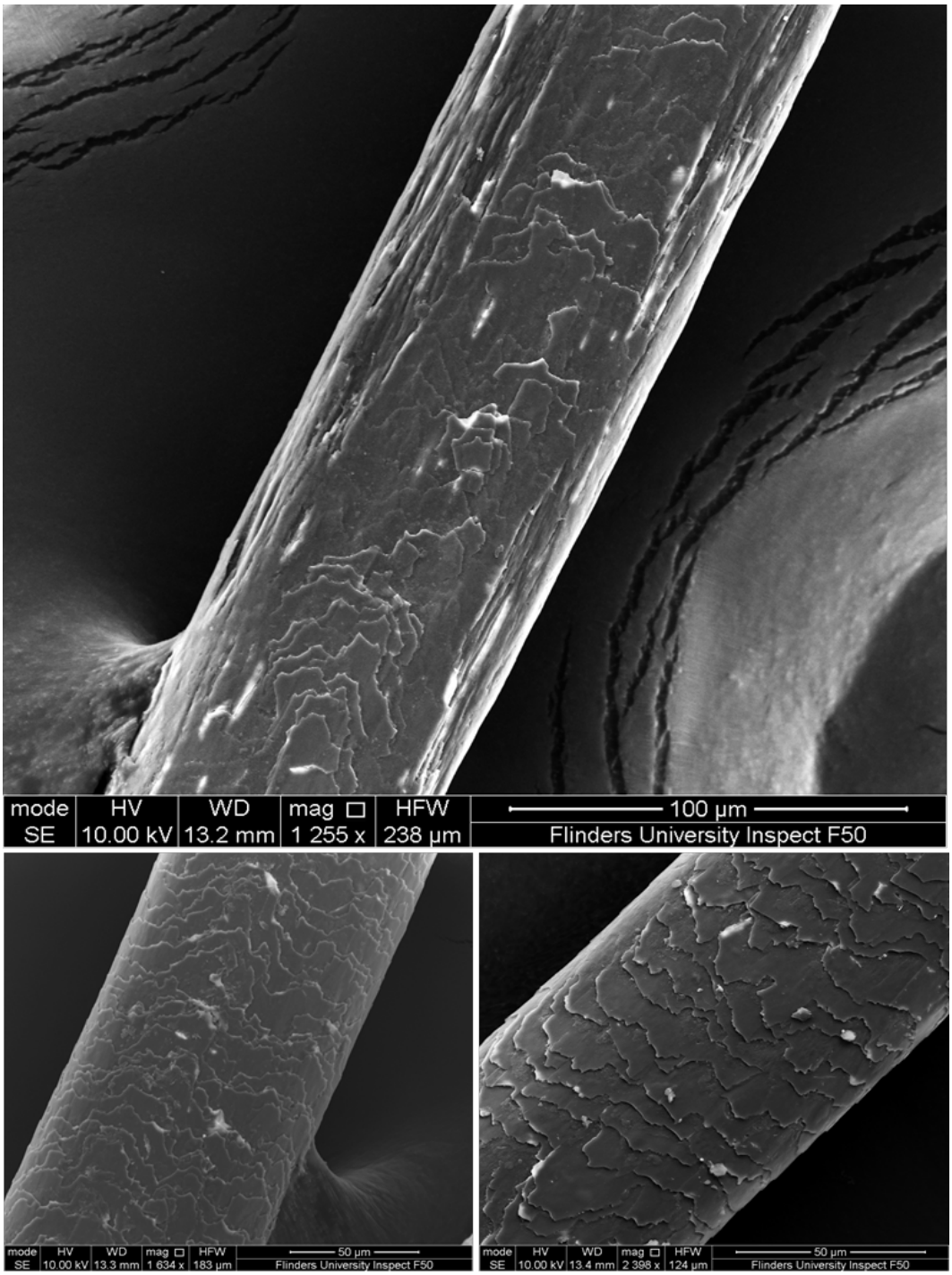


Figure 27: SEM images of human scalp hair that has been bleached with the Olaplex® treatment incorporated, showing the hair after the in-salon treatment (top) and hair treated with 5 additional washes using the Olaplex® No.3 take home treatment, and shampoo and conditioner (bottom left and right). Images taken using Inspect FEI F50 SEM

5.3.3 Assessment of structural damage and repair - single hair assessment

The results presented in section 5.3.2 showed that there was variation in the extent of damage caused by the bleach between hairs within the same sample (refer to Figure 25-Figure 27). This inconsistency between hairs undergoing the same treatment could be the result of the bleach mixture not being evenly distributed across the lock of hair causing varying levels of damage. It also may be due to the nature of hair being non-homogenous and the level of damage to each specific shaft of hair prior to any treatments is unknown. To determine the true extent of damage to a specific shaft of hair, three individual strands of hair (untreated) were mounted onto pieces of silicon wafer and imaged using SEM. These individual hairs with then treated with a) the Olaplex® in-salon treatments (No. 1 and No. 2); b) bleaching treatment; and c) bleaching treatment with Olaplex® incorporated into the bleach. Following treatment, the hairs were re-imaged. All other samples analysed by SEM presented in this thesis were sputter coated with gold prior to SEM analysis, which enable fine detail of the cuticle and the shredding cortex to be viewed. Unlike typical SEM measurements which involve mounting the sample then coating with a conductive film, the three untreated hair samples (i.e. prior to cosmetic treatment) could not be coated prior to SEM as this would prevent the bleach and Olaplex® treatments from working effectively. Similarly, after cosmetic treatment the hair was not coated prior to analysis for consistency. Since there was no coating over the hair, the ion beam energy was reduced from 10 kV to 2 kV as the hair was being damaged at higher energy levels. Furthermore, charging was observed at the higher energy level (refer to Figure 46 in the Appendix). Despite the decrease in ion beam energy, a small amount of charging was observed at the 2kV energy level, making the strands of hair appear to glow.

For hair strand treated with Olaplex® in-salon treatments (No. 1 and No. 2), there was no observable difference in the structure or appearance of the hair before and after treatment (refer to Figure 28). This concurs with the previous findings in section 5.3.2. The SEM analysis of the bleached hair (post bleach-treatment), is presented in Figure 29. As can be seen, charging was observed for this sample and little detail could be observed on the centre of this strand. However, the cuticle scale pattern can be seen along the edges of the shaft. There is also what appears to be keratin flakes can be seen surrounding the hair strand. Whilst it is possible that these particles could be due to the bleach, this was considered unlikely as other bleached samples that were imaged did not contain this

deposit, and all samples underwent the same washing procedure. It is unclear as to why this strand had such a large amount of charging compared to the others. For the hair that was treated with the bleach and the Olaplex® in-salon treatments (No. 1 and No. 2), the hair after treatment showed that all the cuticle scales were still intact (refer to Figure 30). There was not an observable difference between the three treated hairs, and hence analysing single strands of hair before and after treatments did not provide any additional information on how the bleach and Olaplex® treatment affected the hair. Unless alternative imaging techniques, such as environmental SEM (which does not require analysis to be performed under vacuum), are used, coating the hair prior to analysis is essential for viewing the finer details in the hair, and preventing the hair from charging or being damaged during analysis.

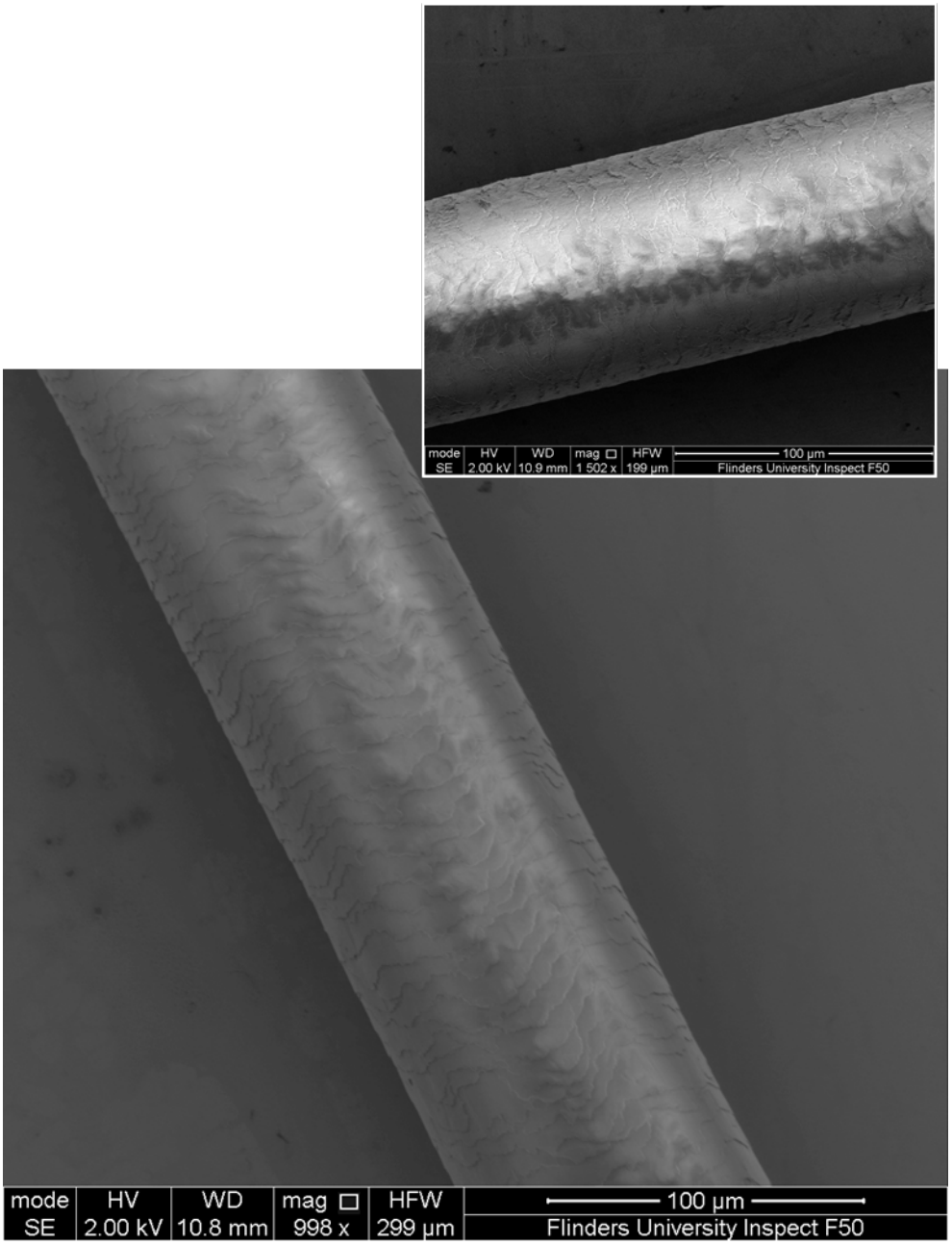


Figure 28: SEM images of healthy, untreated human scalp hair (top) and hair treated with the in-salon Olaplex® treatments, No. 1 and No. 2 (bottom). This single strand of hair was imaged before and after treatment, with the treatment taking place on the SEM stub on which the hair was mounted. Images taken using Inspect FEI F50 SEM

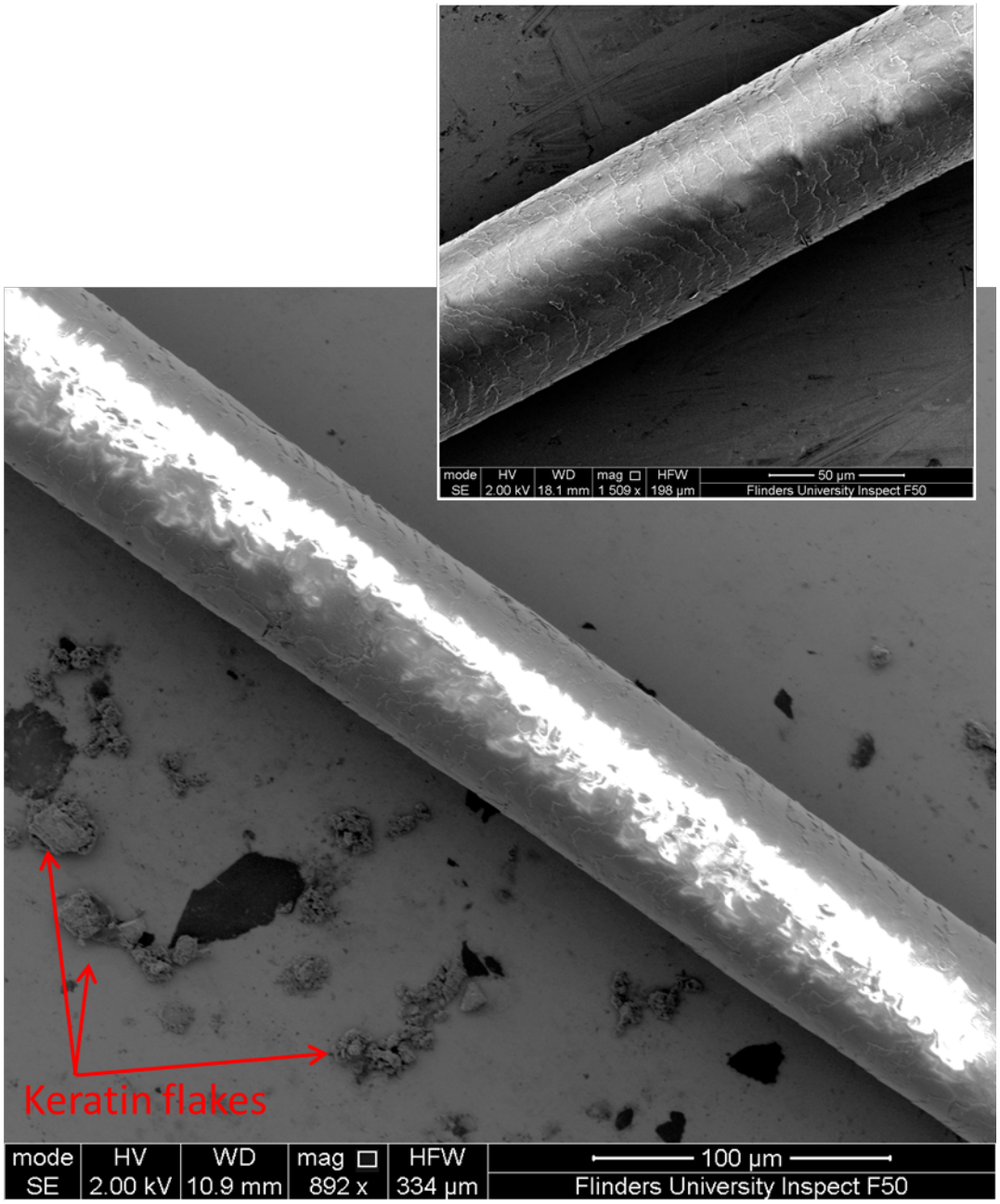


Figure 29: SEM images of healthy, untreated human scalp hair (top) and hair treated with bleach (bottom). This single strand of hair was imaged before and after treatment, with the treatment taking place on the SEM stub on which the hair was mounted. Images taken using Inspect FEI F50 SEM

Figure 30: SEM images of healthy, untreated human scalp hair (top) and hair treated with Olaplex® incorporated into a bleach treatment (bottom). This single strand of hair was imaged before and after treatment, with the treatment taking place on the SEM stub on which the hair was mounted. Images taken using Inspect FEI F50 SEM

5.3.4 Detection of hair treatment using FT-IR and ATR

Strands of hair that had been treated with a) the Olaplex® in-salon treatments (No. 1 and No. 2); b) bleaching treatment; c) bleaching treatment with Olaplex® incorporated into the bleach; and d) untreated, were collected and analysed using Fourier-transform infrared spectrometry (FT-IR). This was done to determine if the various cosmetic treatments could be detected. A study by Witt *et al.* [177] has demonstrated the ability to differentiate between hairs that have been treated with oxidative treatments, and those that have not, using fluorescence microscopy. Using the FT-IR, there was no observable difference between the untreated hair and the hair treated with the Olaplex®. For the samples that had been bleached, both with and without the Olaplex®, then was an additional peak at ~1040 cm^{-1}, typical of a cysteic acid residue [178] (the oxidation products of cysteine) which is indicates oxidation has occurred (refer to Figure 31). ATR analysis was also performed on untreated hairs and hair that had been bleached. Similar to the results presented for FT-IR, a peak at ~1040 cm^{-1} was present in the bleached sample but not the untreated sample, indicating oxidation (refer to Figure 32). Since ATR is a surface analysis technique, it is able to show that the impact of the bleach is at the surface of the hair. While it was possible to differentiate between hairs that have undergone bleach treatments and those that have not, it was not possible to determine whether the hairs had been treated with Olaplex®.

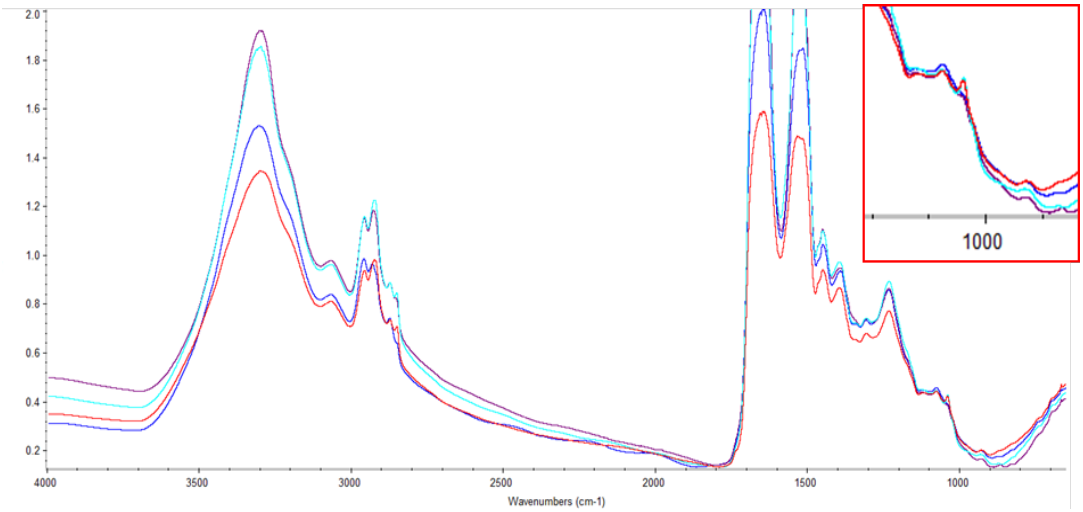


Figure 31: FT-IR spectra of untreated hair (dark blue), and hair treated with Olaplex® in-salon treatments (purple), bleach (red) and bleach with Olaplex® (light blue). Analysis performed on Nicolet™ Nexus 870 FT-IR, coupled with Nicolet™ Continuum™ FT-IR microscope

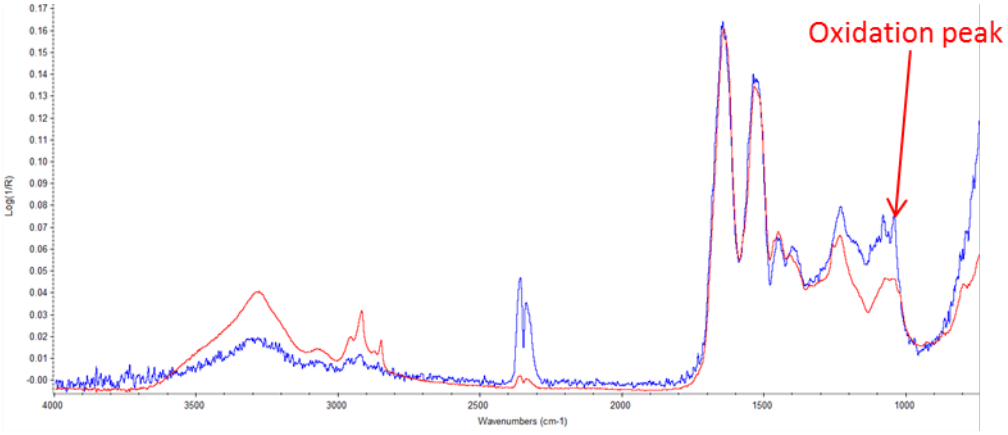


Figure 32: ATR spectra of untreated hair (red) and bleached hair (blue). Analysis performed on ATR was also performed on the Nicolet™ Nexus 870 FT-IR, coupled with Nicolet™ Continuum™ FT-IR microscope using a Germanium ATR crystal

5.3 Conclusions

This chapter investigated i) the effect Olaplex® has on detectable drug concentration, ii) the effect Olaplex® has on the overall structure of the hair shaft (on a microscopic level), and iii) whether or not the effect Olaplex® has can be detected on the hair shaft after use. This is the first time Olaplex® has been investigated in these ways. Olaplex® treatment alone results in reduced detected drug concentration, to a greater extent than the standard shampoo and conditioning which showed little to no decrease in drug content. Bleaching has the largest impact on detected drug concentration, with a substantial decrease seen after just the in-salon treatments, and a gradual decline following with weekly washes. When comparing the samples that have had the stand alone bleach treatment, and the samples that have had the Olaplex® incorporated into the bleach treatment, the use of Olaplex® did not have a observable effect on the drug content in hair, as there was still a large decrease after the in-salon treatments and a gradual decrease with the weekly at-home treatments that followed. However, by the final at-home treatments using the Olaplex®, more of the compounds were able to be detected at higher concentrations. Bleaching had the largest impact in regard to structural damage, with the complete removal of cuticle scales and additional damage to the keratin fibres of the cortex for most of the hairs examined. The use of Olaplex® reduced the damage done by bleach. However, it is not possible to use infrared spectrometry or SEM to determine whether a hair has undergone an Olaplex treatment or another oxidative treatment.

Chapter 6
Artificial incorporation of drugs into hair

This chapter reports the production of a spiked hair standard for the use in R&D projects and analytical method development. The spiked hair standard was achieved through artificial drug incorporation. The results discussed include:

- *the effects of temperature, solvent ratios and cosmetic hair treatments on the quantity of artificial drug incorporation and the structure and integrity of drug incorporated hair*
- *A preliminary investigation into using ToF-SIMS for single hair analysis and drug mapping*

6.1 Introduction

The analysis of drugs from hair samples can provide forensic toxicologists a great deal of information that cannot be gained through the analysis of blood and urine. However, getting hair with known concentrations of drugs present for quality control and quality assurance, as well as method development, validation and accreditation can be quite difficult and expensive, as certified reference materials providers are limited. When using blood and urine samples, drug stocks can be spiked directly to the matrix prior to extraction and analysis, but for hair testing it is a little more complicated as the drug needs to be incorporated into the hair structure, rather than just coating the surface. Some laboratories prefer to source their own reference materials to be used for quality control and method development [179, 180]. Quality control samples should ideally come from authentic hair samples collected from known drug users [7], which introduces a number of other issues. The first being homogeneity of the drug within the hair. Drug distribution will need to be relatively homogenous which can be difficult when attempting to source bulk quantities. The other issue is with availability of case samples (i.e. samples containing target analytes), permissions required to be able to use these samples, and limitations with ethics approvals [7]. For quality assurance and to ensure laboratories are abiding by the recommendations for good practice in hair testing published by the Society of Hair Testing (SoHT) [15, 28, 29], the SoHT conducts annual proficiency testing programmes, where the proficiency hair samples are prepared using hair from known drug users, and all results are published on their website. Purchasing additional proficiency testing samples to use for quality controls is an option available to laboratories, but again the quantity of these samples is limited and samples are not provided for research purposes [7, 28, 29, 179, 180].

These sources of hair reference material are useful for obtaining quality controls, however for method development and R&D purposes, much more hair is required, and hence methods of artificially incorporating drugs into hair, through solvent soaks, are desperately needed and some have been suggested. There are a number of issues that must be taken into account if reference materials are to be produced. The amount that an externally applied drug will bind or incorporated into hair is influenced by a number of factors, some of which also apply for incorporation into hair through ingestion or administration of a drug (refers to Chapter 1.4.7). A number of studies have determined

melanin content to be a significant influencer. Some drugs, like codeine, have a high melanin affinity, meaning a higher concentration of melanin will result in higher concentration of the drug incorporating into the hair [14, 94, 116, 181]. This has been shown to be the case for cocaine and its metabolite benzoylecgonine, where the externally applied analytes resulted in higher concentrations, or higher levels of binding, in Ethnic hair (darker colour, higher melanin content) compared to Caucasoid hair (light brown to blonde in colour, lower melanin content) [182, 183].

The condition of the hair prior to artificial drug incorporation will strongly influence how well the drug will incorporate into the hair. A number of studies, including Blank and Kidwell's studies on external contamination have demonstrated that hair that has been dyed or bleached incorporated externally applied drugs more effectively. It was hypothesised that this phenomenon is due to the damage that occurs to the hair cuticle which helps to facilitate drug incorporation. As the cuticle is quite an effective barrier that restricts drug penetrating into hair shaft, treatments that compromise the cuticle may facilitate incorporation of drug into the shaft [94, 184]. Conversely, Joseph *et al.* [183] demonstrated that bleaching reduced the in-vitro binding of cocaine, due to the bleaching causing a reduction in melanin content.

Due to a number of restrictions with the ethics approval for this project, hair could not be collected from any donor who had illegally taken drugs, or misused prescription drugs. This would have greatly limited the number of drugs and metabolites that could be assessed, since >50% of the target analytes used in this project are either illicit or controlled substances. Additionally, the hair reference material available for purchase also only contains a fraction of the drugs of interest in this project, and the budget for this project also prevented purchase of large enough quantities to complete all the necessary assessments. As such, to complete the method development and validation experiments outlined in Chapters 3-5, reference material containing all 12 drugs of interest needed to be made in-house.

Although several literature articles refer to incorporation of drugs in hair for research purposes, methodology is poorly described. Variable factors such as incubation time and temperature, and whether the hair has previously been cosmetically treated have also not been reported in literature. One article, by Martin *et al.* [133], describes in some detail

the incorporation of hallucinogens in hair. For this project, variations of this method were used to assess levels of drug incorporation under varied conditions, including solvent ratios, temperatures and the effect of incorporation after the hair had been cosmetically treated.

ToF-SIMS analysis was used in an attempt to determine the distribution of incorporated drugs in single strands of hair and SEM analysis was used to determine the structural damage to hair during the incorporation process.

6.2 Experimental

6.2.1 Hair sample collection and preparation

A lock of hair was collected from donor 10 (refer to Chapter 2.2, Table 4, sample 10), and screened to ensure the hair was drug free. The lock was divided into 8 sections, each to be prepared through a different method of artificial drug incorporation, described below. The original method of artificial drug incorporation (refer to Chapter 2.2) was based on a method described by Martin *et al.* [133], which involved hair (1 g) being soaked in a solution of different hallucinogens at concentrations ranging from 1 µg/mL to 20 µg/mL, made up in methanol/dimethyl sulphoxide (DMSO) (1:1 v/v, 20 mL), for 7 days under nitrogen atmosphere, away from light.

6.2.2 Effect of temperature and solvent ratios on incorporation

For 6 of the sectioned locks, 100 mg samples (finely cut into 1-5 mm fragments) were weighed out into new glass specimen containers and washed with methanol (2 mL swirled for 30 seconds), the solvent removed, and the hair left to dry at room temperature. The remaining two section locks were treated prior to washing, and the treatments are discussed in section 6.2.3 below. Subsequently, each of the samples of cut hair were soaked in the standard drug mix solution (1 µg/mL) prepared by dilution in methanol/dimethyl sulphoxide (DMSO) (2 mL) and soaked for 7 days under nitrogen atmosphere and away from light. The MeOH/DMSO solvent ratios and the temperatures at which the samples were stored for the 7 days are presented in Table 28. After 7 days the drug mix was removed, and the artificially incorporated hair samples were washed ten times with methanol (2 mL, swirled for 30 seconds) to ensure any remaining analyte was incorporated into the hair and not coating the surface of the hair. The samples were

then left to dry at room temperature, and 3x20 mg from each sample was weighed out and extracted using the 18-hour incubation method (refer to Chapter 2.3), and analysed using the LC-QTRAP® 4000 (refer to Chapter 2.4.2).

Table 28: Solvent ratios for the MeOH/DMSO drug mix solution (v/v) and storage temperatures (°C) for the assessment of artificial drug incorporation into hair. All samples, were soaked for 7 days, under nitrogen atmosphere, away from light (unless otherwise specified

	Solvent ratios (MeOH/DMSO)	Storage temperature	Other conditions
1	1:1 v/v	Room temperature	N/A
2	1:1 v/v	35 °C (stored in oven)	N/A
3	1:1 v/v	-15 °C (stored in freezer)	N/A
4	1:1 v/v	Room temperature	Stirred (using stirrer bar set up) for the 7 days, exposed to light
5	2:1 v/v	Room temperature	N/A
6	1:2 v/v	Room temperature	N/A
7	1:1 v/v	Room temperature	Treated with Olaplex® No. 1 & 2 (refer to 2.3.2)
8	1:1 v/v	Room temperature	Bleached (refer to section 2.3.2)

6.2.3 Effect of hair treatments (bleach and Olaplex®) on incorporation

Locks 7 and 8 underwent cosmetic treatment prior to artificial drug incorporation. The first lock underwent an Olaplex® treatment (discussed in Chapter 5). Olaplex® No. 1 (7.5 mL) was diluted in water (42.5 mL) and added to the dry lock of hair until saturated and left to stand at room temperature for 5 minutes. Without rinsing, the Olaplex® No. 2 was massaged into the locks and left to stand at room temperature for a further 20 minutes. The amount of Olaplex® No. 2 used was enough to sufficiently cover the entire lock of hair. The hair was then rinsed, washed with Garnier® Fructis fortifying shampoo and conditioner for normal hair, and left to dry at room temperature. The second lock was bleached with a Results brand bleach powder and hydrogen peroxide cream (20 vol., 6 %) treatment. Bleach powder (35 g) was mixed with 20 vol. peroxide cream (70 mL) until it formed a thick cream consistency and it was then added to dry hair. This was left to stand at room temperature for 25 minutes, and was then rinsed, washed with Garnier® Fructis fortifying shampoo and conditioner for normal hair, and left to dry at room temperature.

Following the cosmetic treatments, artificial incorporation was undertaken through the process described in section 6.2.2 above, using the 1:1 v/v MeOH/DMSO soak at room

temperature, followed by the wash and extraction procedures. Samples were analysed using the LC-QTRAP® 4000 (refer to Chapter 2.4.2).

6.2.4 Assessment of damage done to hair through spiking process

Fibres of hair (6-8) were collected prior to treatment, and after artificial drug incorporation using the 1:1 v/v (MeOH/DMSO) at room temperature method analysed through SEM (refer to Chapter 2.4.4 for details). For this section, 0.5-1 cm lengths were used, instead on the 1-5 mm fragments used in the previous section, to make SEM analysis easier, i.e. longer strands are easier to mount, and gives greater surface area to examine.

6.2.5 Single hair analysis and drug mapping

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) was performed on drug-free (blank) hair (sample 10), artificially incorporated hair (using the 1:1 v/v (MeOH/DMSO) at room temperature method) and an authentic hair sample known to contain oxazepam and temazepam (refer to Chapter 2.2, Table 4, sample 11). In preparation for analysis single strands of hair were mounted via 3 methods below. Each hair sample was mounted in duplicate, and one of the strands was then scraped using a new, clean razor blade, being held at 45° to the hair and dragged along the shaft, being careful to apply even pressure along the hair shaft and not to 'nick' the hair. This was to expose the inner cortex of the hair. The other mounted strands for each of the sample types were left un-scraped. For ToF-SIMS instrumental details refer to Chapter 2.4.5.

Mounting method 1

Single strands of the blank, incorporated, and authentic hairs were laid flat along a piece of double-sided tape (Sellotape), adhered to a microscope slide.

Mounting method 2

Single strands of the blank, incorporated, and authentic hairs were mounted onto the adhesive strip of 3M post-it notes.

Mounting method 3

Single strands of the blank, incorporated, and authentic hairs were mounted onto a 2x2 cm piece of silicon wafer, using carbon paint as an adhesive. The hair was tightly

wrapped around the wafer and secured on the underside (the rough side) of the wafer with the carbon paint (refer to Figure 33 left). This leaves the desired section of the hair strand running along the top (the shiny side) of the silicon wafer (refer to Figure 33 right). Securing the hair underneath on the rough side makes the scraping process easier as the blade is scraping along a smooth surface, the hair is less likely to slip away from the adhesive during the scrapping and hair is easier to view under the microscope when it is on the shiny side.

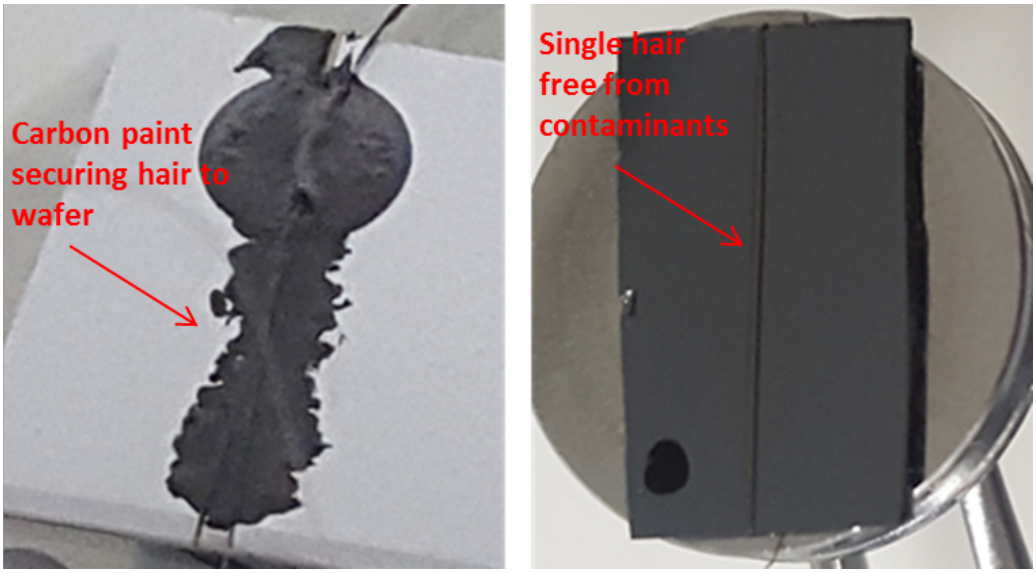


Figure 33: Images of a single strand of hair mounted onto a piece of silicon wafer, showing the underside of the wafer where the hair is secured with carbon tape (left) and the topside of the wafer with the exposed strand of hair running along the surface (right)

6.3 Results and discussion

6.3.1 Artificial incorporation of drugs into hair

The concentrations of each analyte in hair after the various incorporation treatments are presented in Table 29. In terms of variation in temperature 9 of the 12 target analytes had higher concentrations in the samples in which incorporation occurred at 35 °C, and the lowest concentrations after incorporation at -15 °C. Morphine and codeine were the main exceptions with the highest concentrations occurring in the lower temperature samples (incorporated at -15 °C), and the lowest at 35 °C. For oxazepam, incorporation at room temperature and at 35 °C produced similar concentrations in the hair. Apart from these exceptions, the level of incorporation increases as the temperature increases.

Incorporation using the 2:1 MeOH/DMSO ratio resulted in higher concentrations in 10 of the 12 target analytes compared to the 1:1 MeOH/DMSO samples (refer to Table 29). Amphetamine and methamphetamine were the two exceptions, where the incorporation using 2:1 MeOH/DMSO resulted in concentrations 25 % and 38 % lower, respectively, than the sample incorporated using 1:1 MeOH/DMSO. An issue with the 2:1 ratio method is there were higher levels of analyte being detected in the wash. While most were still below the limit of quantification (<LOQ) methamphetamine was detected at a high concentration of 47 pg/mg. This indicates that the concentration of methamphetamine in the hair may be lower as there may still be a significant amount left coating the hair. This is further supported by the fact that methamphetamine could not be detected at all in one of the 3 replicates. Through using the 1:2 MeOH/DMSO ratio method, the calculated concentrations were lower in the hair extracts compared to the 1:1 ratio method for all drugs, with methamphetamine not being detected at all. Once again, more of the drugs were present in the wash (still below LOQ). For both of the altered ratio methods, a more thorough wash process is needed.

The hair that was constantly stirred for the 7 days resulted in the highest concentration for all analytes compared to all of the other incorporation conditions (refer to Table 29). However, on observation the hair appeared quite damaged with the hair being ground down by the stirrer bar. This would not be a good method to use, regardless of the incorporated concentration, as it will not be an accurate representation of hair in normal situations. The damage to the hair may also be allowing the analytes to be extracted more

easily, and hence these results may not be an accurate representation of how well the drug has incorporated compared to the other methods of incorporation.

The hair treated with Olaplex® prior to incorporation had a much lower level of incorporation compared to the untreated hair, except for the benzodiazepines (refer to Table 29). The largest reduction in concentration occurred in the stimulants, amphetamine, methamphetamine and MDMA. Methamphetamine could not be detected at all, and amphetamine, while still being detected, was below the limit of quantification (<LOQ). MDMA was within the quantifiable range, but only had a concentration of 37 pg/mg, whereas the untreated hair had a concentration of 250 pg/mg. It has been hypothesised that these drugs are either not incorporating far enough into the cortex and being removed during the methanol washes, or they are binding strongly to the hair and not extracting during the incubation. The amine functional groups in the stimulants could potentially be interacting with the bound maleate group arising from the bis-aminopropyl diglycol dimaleate in the Olaplex® (refer to Figure 34), similar to the way the diamine interacts with the bound maleate within the Olaplex® structure, preventing the drugs from being extracted. In contrast, the benzodiazepines, temazepam, oxazepam, nordiazepam and diazepam, all had higher extracted concentrations in the Olaplex® treated hair than the untreated hair by 9 %, 7 %, 26 % and 43 % respectively. In this situation, the Olaplex® could be acting as a surfactant, drawing the analytes (which would be uncharged at neutral pH) out of the hair and assisting with the extraction processes for these drugs. This can also be seen in Chapter 5.3.1 (refer Figure 21) with the increase of temazepam concentration with the number of washes, for both the Olaplex® treated hair and the normal shampoo and conditioned hair, and to some extent both the bleach treated samples. The Olaplex® and the conditioner may be drawing the temazepam out of the hair assisting in the extraction. However, this was not the case for the other benzodiazepines. For the incorporation of drugs into hair previously treated with Olaplex®, the level of incorporation compared to the incorporation into the untreated hair seems to be inversely related to the pK_a (refer to Table 30). The analytes with lower pK_a values, i.e the benzodiazepines, had a higher level of incorporation and the analytes with the higher pK_a values, i.e the amphetamine-like drugs had a lower level of incorporation.

Table 29: Concentrations of the target analytes (pg/mg ± 1 SD) found in the final wash and in hair after artificial incorporation. Hair samples extracted using the 18 hour incubation in methanol (where n=1 for the wash, and n=3 for hair)

		AMP	MDMA	METH	MOR	COD	TEM	OXAZ	NOR	DIAZ	MET	COC	OXY
1:1 v/v, r.t. (control)	Wash		<LOQ		<LOQ	<LOQ							
	In hair (Average ± SD)	68.6 ± 5.05	249 ± 5.71	43.6 ± 3.04	162 ± 6.19	114 ± 11.1	107 ± 7.42	129 ± 6.31	38.6 ± 3.74	29.8 ± 1.19	65.9 ± 11.1	26.5 ± 2.47	78.9 ± 5.74
1:1 v/v, 35 °C	Wash	<LOQ	<LOQ		<LOQ	<LOQ			<LOQ		<LOQ		
	In hair (Average ± SD)	76.5 ± 8.37	265 ± 26.7	121 ± 66.9	134 ± 14.6	106 ± 8.41	114 ± 10.6	127 ± 12.2	42.6 ± 7.63	35.3 ± 6.83	74.8 ± 10.1	28.7 ± 4.35	81.5 ± 9.32
1:1 v/v, -15 °C	Wash	<LOQ	<LOQ		<LOQ								
	In hair (Average ± SD)	48.7 ± 2.52	230 ± 10.0	65.0 ± 19.2	177 ± 9.19	127 ± 10.9	73.3 ± 10.4	96.8 ± 9.93	30.2 ± 1.65	<LOQ	53.4 ± 0.445	22.4 ± 2.04	74.0 ± 6.34
1:1 v/v, r.t., stirred	Wash	<LOQ	<LOQ		<LOQ	<LOQ	<LOQ		<LOQ				<LOQ
	In hair (Average ± SD)	280 ± 26.8	390 ± 35.0	203 ± 21.5	255 ± 21.3	233 ± 23.5	237 ± 19.9	137 ± 15.3	65.4 ± 5.21	59.7 ± 7.14	75.3 ± 5.53	48.8 ± 5.49	108 ± 10.5
2:1 v/v, r.t.	Wash	<LOQ	<LOQ	46.9	<LOQ	<LOQ		<LOQ	<LOQ				<LOQ
	In hair (Average ± SD)	51.4 ± 3.03	296 ± 3.24	27.0 ± 24.1	283 ± 4.93	201 ± 11.5	191 ± 4.59	259 ± 14.4	71.2 ± 3.44	60.8 ± 6.96	71.9 ± 9.02	47.4 ± 4.67	127 ± 12.2
1:2 v/v, r.t.	Wash	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	In hair (Average ± SD)	27.3 ± 1.12	170 ± 4.49		50.1 ± 3.32	37.4 ± 3.37	36.6 ± 2.95	41.2 ± 2.15	<LOQ	<LOQ	22.6 ± 2.04	<LOQ	31.2 ± 2.13
1:1 v/v, r.t., Olaplex®	Wash				<LOQ								
	In hair (Average ± SD)	<LOQ	36.7 ± 1.35		116 ± 3.25	109 ± 8.61	117 ± 4.90	138 ± 1.95	49.4 ± 0.108	43.4 ± 2.85	15.9 ± 1.93	20.3 ± 0.756	34.6 ± 0.887
1:1 v/v, r.t., Bleached	Wash		<LOQ		<LOQ	<LOQ					<LOQ	<LOQ	<LOQ
	In hair (Average ± SD)	95.7 ± 7.21	334 ± 13.3	76.5 ± 2.14	119 ± 3.40	83.6 ± 3.60	133 ± 9.57	147 ± 7.90	52.0 ± 1.37	46.1 ± 1.31	57.8 ± 3.99	31.8 ± 1.97	83.3 ± 3.22

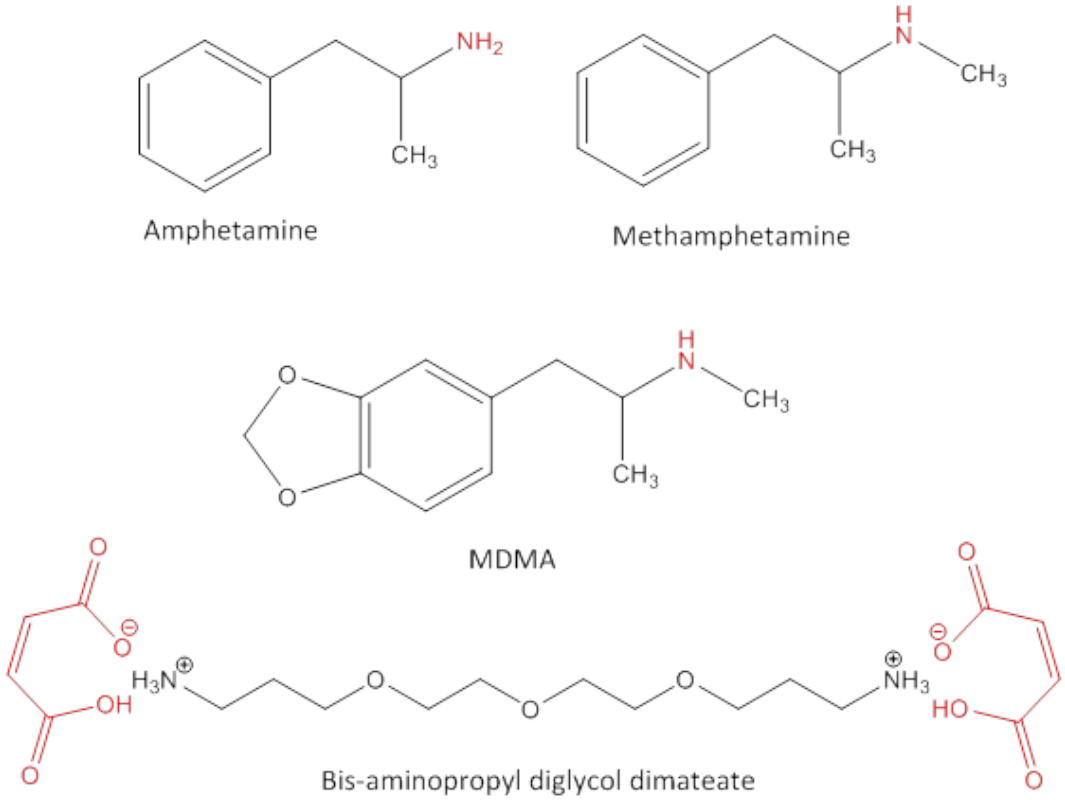


Figure 34: Structures of amphetamine, methamphetamine, MDMA and bis-aminopropyl diglycol dimaleate (Olaplex®)

Table 30: pK_a values of each target analyte (in ascending order of pK_a) with the concentration of each analyte in the Olaplex® treated hair, presented as a percentage of the concentration in the untreated hair [185, 186]

Target Analyte	pK_a	%
TEM	1.3	109
OXAZ	1.7	107
DIAZ	3.4	143
NOR	3.5	126
COD	8.2	95.6
MOR	8.2	71.6
OXY	8.5	44.3
MET	8.6	24.2
COC	8.6	74.1
AMP	9.9	>17.4
MDMA	9.9	14.9
METH	9.9	0

The hair that was bleached prior to incorporation resulted in higher concentrations of most drugs when compared to untreated incorporated hair. Methamphetamine was the most effected, with the concentration being 73 % higher than in the untreated hair. This is consistent with previous studies that indicate bleached hair has a higher level of drug binding [184]. However, the opioids, morphine, codeine and methadone, had lower concentrations in the bleached sample. This is most likely due to the compounds being washed out during the methanol wash cycles.

For both the bleached hair and stirred hair, the high level of incorporation is likely due to the damage done the structure of hair. For the stirred sample, the hair was being ground down into finer pieces, increasing the surface area of each segment, allowing for more uptake. For the bleached sample, the cosmetic treatment causes a severe amount of damage to the hair (refer to Chapter 5.3.2, Figure 25 and Figure 26, for SEM images of bleached hair), removing the cuticle scale and exposing the inner cortex. Hair-shaft damage increases drug incorporation because drugs must first penetrate the cuticle, and this penetration is aided by the damage. This could also explain why the hair treated with the Olaplex® had a lower level of incorporation. The Olaplex® treatment (refer to Chapter 5) is marketed as a restorative treatment, that will re-build broken disulphide bonds within the hair. This may make it harder for the drugs to penetrate the cuticle.

6.3.2 Assessment of damage done to hair through spiking process

Microscopic analysis of the hair before and after being artificially incorporated with drugs, via the 1:1 v/v MeOH/DMSO solvent soak at room temperature, showed that the hair was being damaged during the incorporation process. The level of damage varied between the strands of hair within the sample, which may be due to the nature of hair being non-homogenous. Furthermore, the level of damage to each specific shaft of hair prior to any treatments was unknown. SEM shows a single hair prior to drug incorporation (Figure 35 (top)) and the hair after drug incorporation (Figure 35 (bottom)). As can be seen, prior to incorporation all the cuticle scales are still intact, whereas after incorporation a large portion of the cuticle scales have been removed, and in some sections the inner cortex had been exposed (refer to Figure 36 for enlarged image of the exposed cortex). The strand of hair presented in Figure 35 and Figure 36 is an example of the most severe damage observed, this type of damage was observed in approximately 40% of the specimens examined. All other artificially incorporated hair strands examined were damaged, however the damage was less severe. In these cases, only some of the cuticle scales had been removed. There was no evidence of the inner cortex being exposed (refer to Figure 37). Hence, the damaged hair strand presented in Figure 35 and Figure 36 may have already been damaged prior to drug incorporation which would exacerbate the damage that occurred during the incorporation process. To determine the true extent of damage to a specific shaft of hair it may be nesseccesary to mount a section of untreated hair to image, and artificially incorporate the individual hairs while on the mount, with SEM analysis taking place before and after drug incorporation. However, since the incorporation process consists of a solvent soak taking place over 7 days, under nitrogen, treating the hairs while mounted was unable to be performed. Regardless, potential damage to the strands of hair throughout the incorporation process should be considered when using artificially spiked hairs for method development purposes.

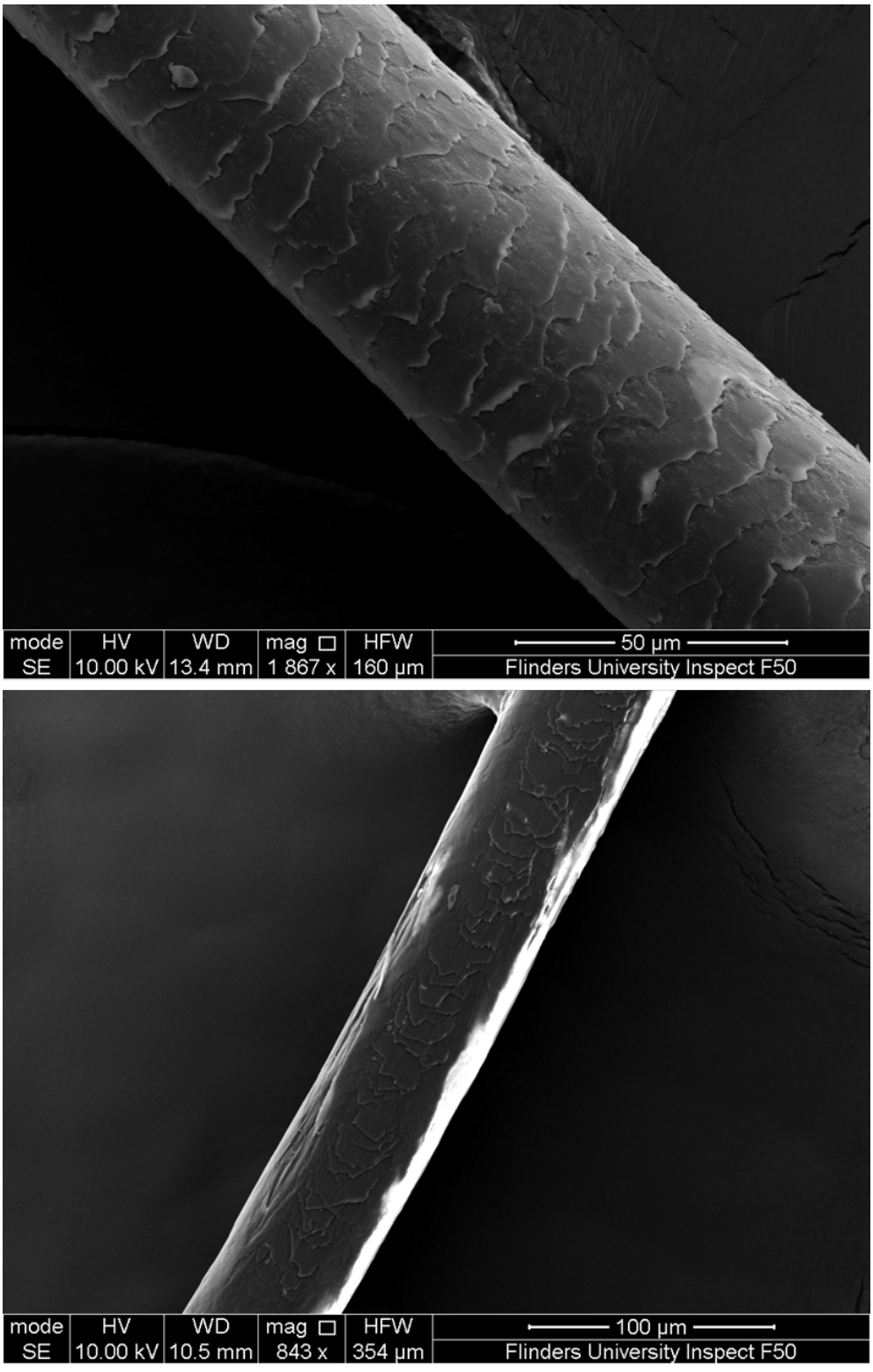


Figure 35: SEM images of healthy, untreated human scalp hair (top), and hair that has been artificially incorporated with drugs using a 1:1 v/v MeOH/DMSO soak, at room temperature for 7 days (bottom). Images taken using Inspect FEI F50 SEM

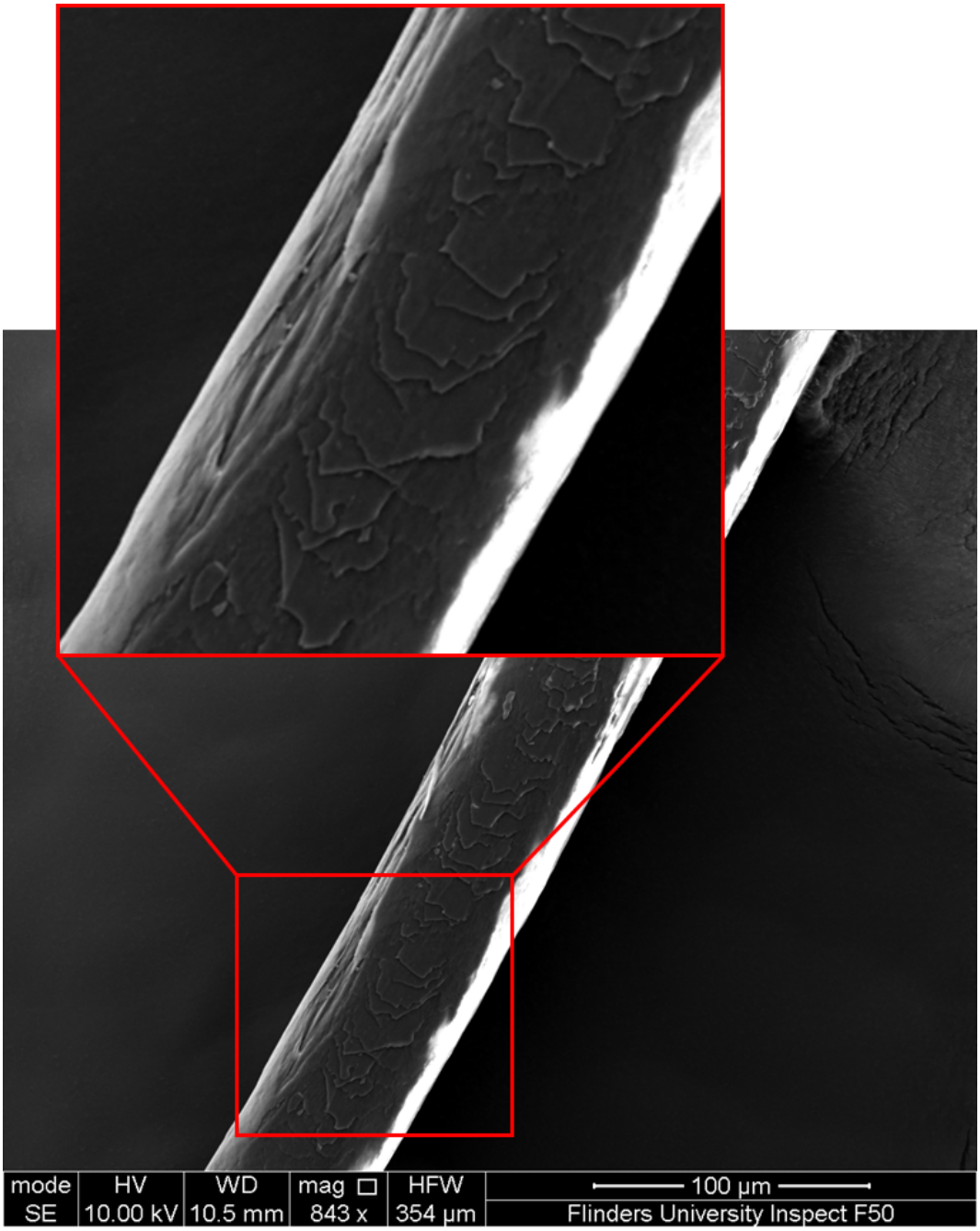


Figure 36: SEM image of a strand of hair after being artificially incorporated with drugs using a 1:1 v/v MeOH/DMSO soak, at room temperature for 7 days, with enlarged section of the image showing the exposed cortex. Images taken using Inspect FEI F50 SEM

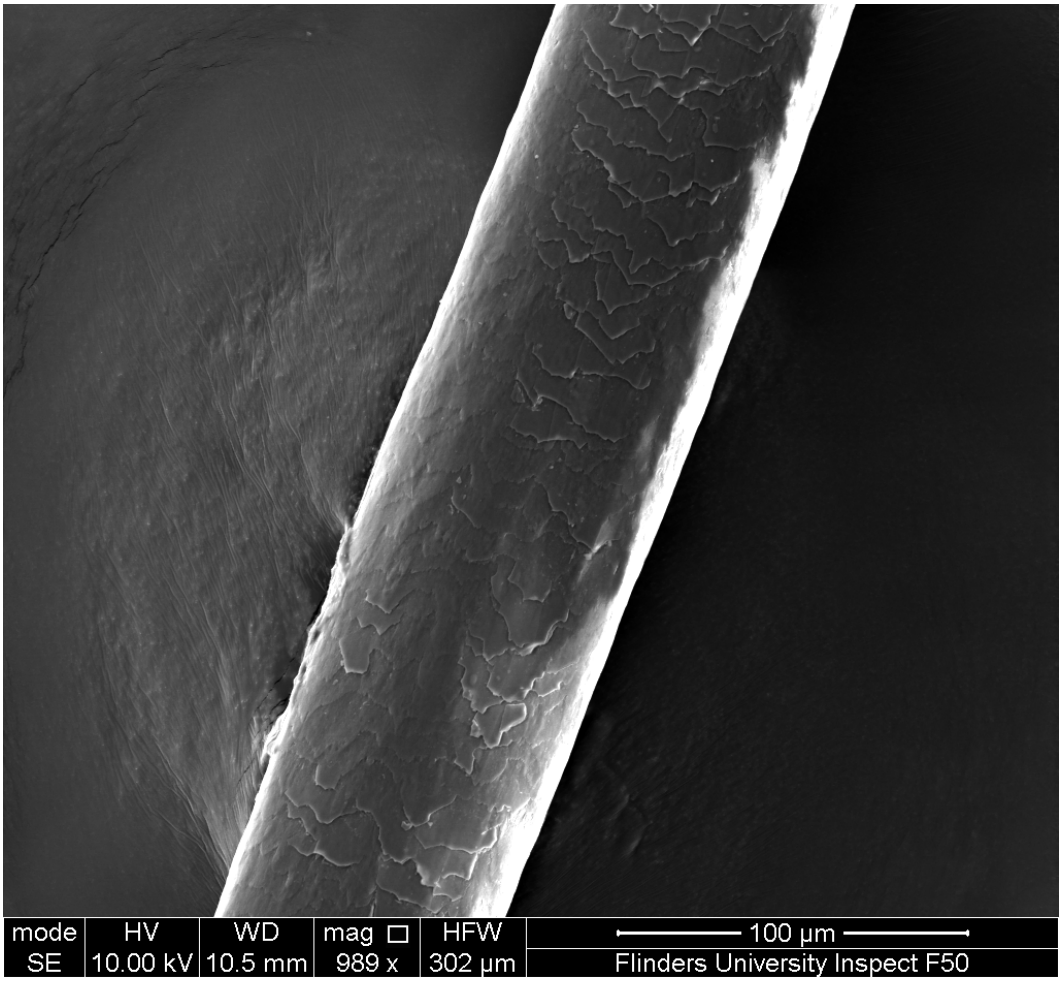


Figure 37: SEM image of a strand of hair after being artificially incorporated with drugs using a 1:1 v/v MeOH/DMSO soak, at room temperature for 7 days, representing the least amount of structural damage that occurred during incorporation process. Images taken using Inspect FEI F50 SEM

6.3.3 Investigations into the location and distribution of the artificially incorporated drugs

It is important that the artificially incorporated hair samples prepared accurately mimic authentic drug-containing hair in terms of the drug distribution across the radius of the hair shaft. Ideally, the artificially incorporated hair samples will have the same distribution of drugs as those of authentic samples. This is important because if the artificially incorporated drugs are only located on the surface of the cuticle, for example, method development using the artificial samples may indicate a greater extraction efficiency than that would normally be achievable with authentic, unadulterated samples. Examination of bulk samples using LC-MS is unable to establish the location of the drug within the hair structure, and the effect this may have on the extraction, thus microscopic analytical techniques are required to understand the distribution of the drugs within the hair shaft. The location and distribution of the drugs in hair can be difficult to assess, but single hair analysis and drug mapping has been reported for this. Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry and Infrared (IR) microscopy have previously been used for this purpose. Numerous studies involving MALDI have reported successful detection of cocaine and its metabolites, methamphetamine, ketamine, zolpidem, olanzapine, and THC and its metabolites [75-87]. Very few studies have been conducted using IR [88, 89], but highlight the various limitations associated with IR for single hair analysis. Infrared microscopy is only useful for detecting chronic use of drugs as such a small portion of hair is used and this analytical technique is very labour intensive and requires a lot of sample preparation. Additionally, given the complex mixture of drugs used, and their similar chemical structures, IR is unable to be used in this current study as it would be very difficult to deconvolute the multiple overlapping spectral peaks from each of the drugs to attribute a single drug. Similarly, whilst there was a MALDI mass spectrometer available, it did not have high resolution mapping capabilities. Therefore, preliminary studies were performed using Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) to see if this could also be used. One study by Flinders *et al.* [81] has already shown successful detection for cocaine, benzoylecgonine and methadone in hair using ToF-SIMS.

The sample types used for this analysis included blank (drug free) hair, authentic hair containing oxazepam and temazepam and the artificially incorporated hair. Since the authentic hair sample only contained oxazepam and temazepam, these were the only

target analytes for this analysis (molecular ion occurring at 287 *m/z* for oxazepam and 301 *m/z* for temazepam). For the reference spectra for both oxazepam and temazepam, refer to Figure 47, in the Appendix.

In preparation for ToF-SIMS analysis 3 methods of mounting the hair were assessed. The first used double-sided tape to secure a length of hair (approximately 2 cm). During ToF-SIMS analysis it was found that there was evidence of polydimethylsiloxane (PDMS) contamination from the tape adhesive, resulting in the characteristic "silicone" peaks including 73 and 147 *m/z*. The PDMS signals were suppressing or masking any signals from the target analytes. In order for the analysis to work effectively, the hair would need to be mounted onto a surface that does not contain PDMS. Most adhesives contain PDMS, however the adhesive used for 3M post-it notes does not and are routinely used by the operators of the ToF-SIMS without issues. Hence, the adhesive strip on this specific brand of post-it notes was used. However, the strands of hair did not adhere as well as with the double-sided tape which made scraping more difficult. The strands of hair also twisted during and after scraping, which meant the sections of the exposed cortex could not be analysed. For this reason, the hair mounted via this method was not analysed.

The final method of mounting trialled, involved the hair strand being wrapped around a piece of silicon wafer and secured on the underside with carbon paint. Once the carbon paint was dry, the hair could be easily scraped, without the strand moving or twisting. However, during the analysis, the PDMS signal was still present in all the hair samples. The intensity of the PDMS signals was less than when using the double-sided tape but still resulted in the target analytes being supressed. Oxazepam (287 *m/z*) and temazepam (301 *m/z*) should have been present consistently along the shaft of hair for both the artificially incorporated sample and the authentic sample, since both drugs were consumed on a regular basis (1-2 times a week) by the donor. However, neither of the drugs could be detected (refer to Figure 38, and Figure 48 in the Appendix). The PDMS was present in all the hair samples, blank, authentic and artificial (refer to Figure 49 to Figure 52 in the Appendix), even after scraping to reveal the inner cortex and taking care not to let the mounted hair come in contact with any PDMS containing substances. This indicates the PDMS is incorporated onto the surface of the hair, and has penetrated down into the cortex. It is likely the PDMS is from regular use of shampoos and conditioners containing PDMS and other siloxane substances. While the study by Flinders *et al.* [81]

did have a relatively high 147 *m/z* signal (refer to Figure 39), indicating presence of siloxanes, it did not mask the target analytes, with cocaine and benzoylecgonine being detected (refer to Figure 40). The differences between the two studies could be due to variation in the analytical method used, or variation in the donors hair, i.e. the donors for this study have higher levels of PDMS and siloxanes in their hair compared to those in Flinders' study. Since neither of the target analytes could be detected in any of the hair samples in this study as a result of the PDMS signal, ToF-SIMS is not an appropriate for drug mapping and single hair analysis at this point. More work is needed to determine possible methods of reducing the PDMS signal.

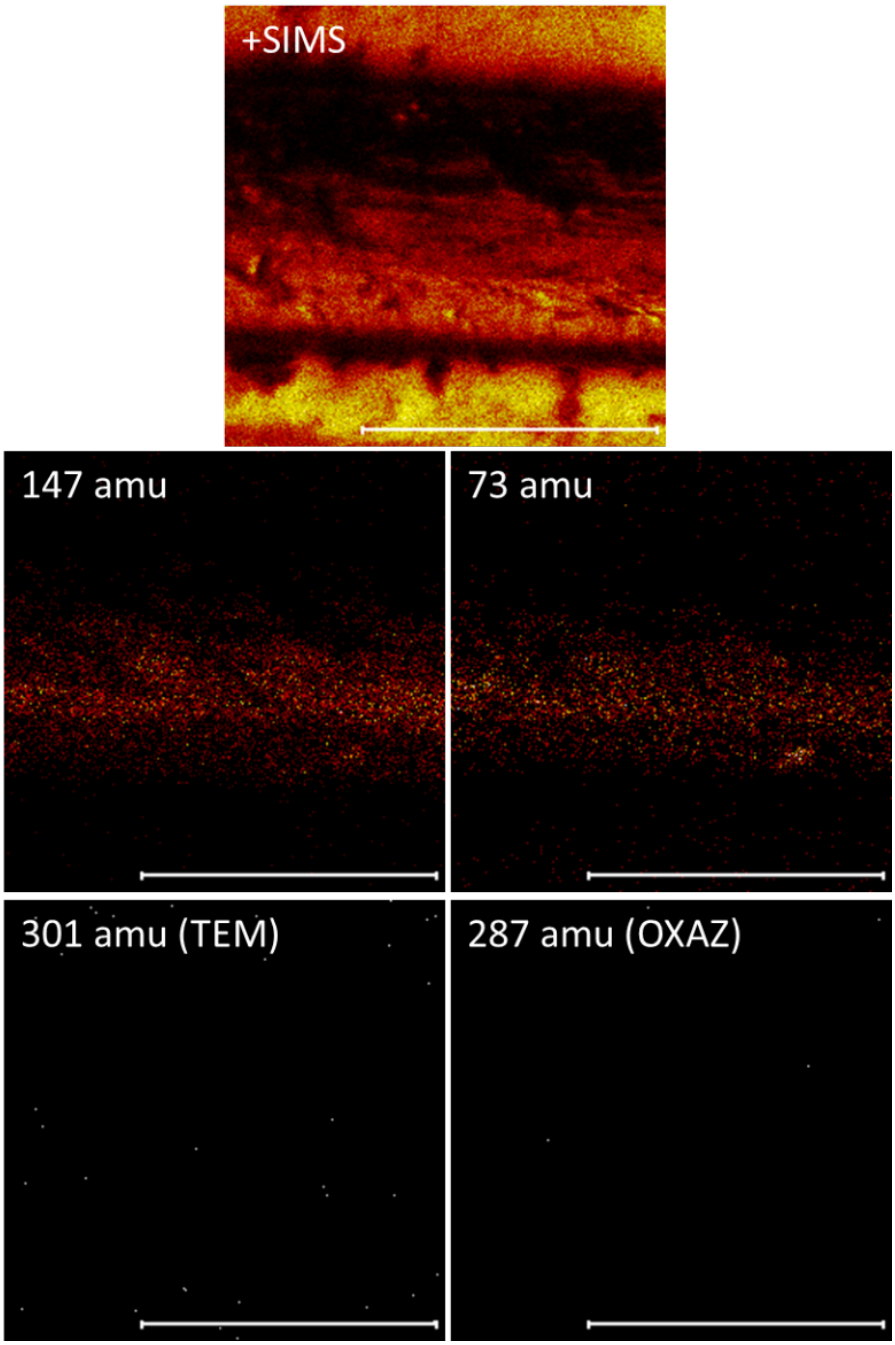


Figure 38: ToF-SIMS images for the artificially drug incorporated sample (scraped to expose cortex) of total ion count (top), 147 and 73 ions indicating presence of PDMS (middle, left and right), 301 ion indicating temazepam (bottom, left) and 287 ion indicating oxazepam (bottom, right)

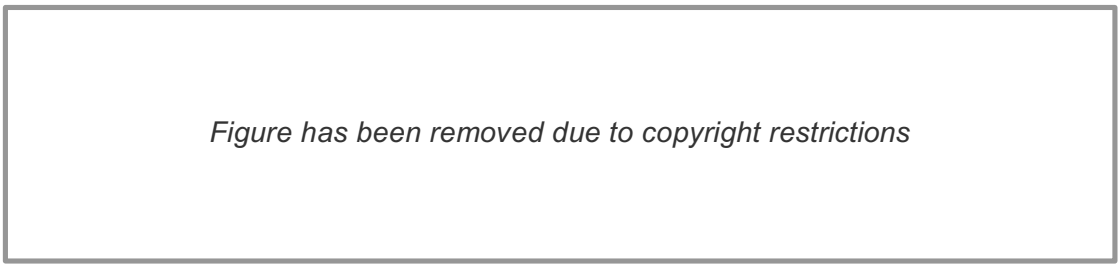


Figure 39: Average positive ion spectrum from drug users hair (Scale 1 mm), as reported by Flinders *et al.* [81]

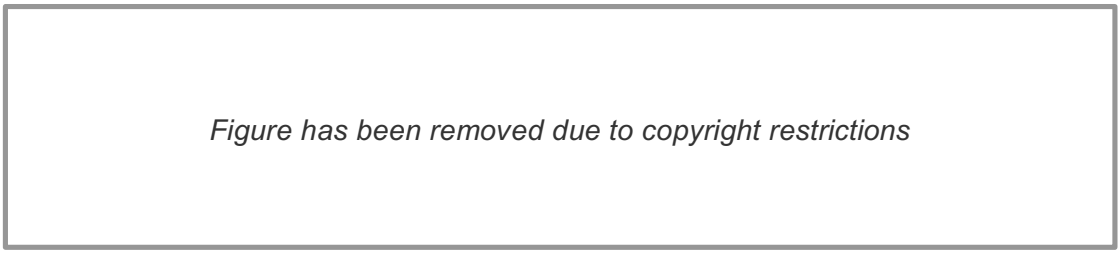


Figure 40: MetA-SIMS images of a longitudinal sectioned cocaine user hair sample showing A) the total ion count, B) benzoylecgonine at m/z 290 and C) cocaine at m/z 304 (Scale 10 μm). As reported by Flinders *et al.* [81]

6.4 Conclusions

This chapter investigated the effects of temperature, solvent ratios and prior cosmetic treatments of the hair have on the quantity of drug that is incorporated into the hair shaft. This work present new insights on how effectively drugs can be incorporated into hair, and how procedures can be altered to suite the requirements of the reference material being produced. In summary, artificial incorporation using the solvent soak produced higher detected concentrations of the target analytes in the hair, when incubating at 35 °C for the 7-day soak and using a 2:1 v/v MeOH/DMSO for the solvent. The hair bleached prior to artificial incorporation also resulted in higher concentrations in the hair extracts, which supports previous work. The hair treated with Olaplex prior to incorporation had the opposite effect and prevented some of the drugs from incorporating, or from being extracted properly. This work also showed a major flaw in the reference materials produced through artificial incorporation via solvent soak, that has not previously been reported. Through microscopic analysis, damage to the hair during the drug incorporation process was observed. For a larger proportion of the hairs assessed the damage was minimal. However, more extensive damage was also observed with numerous cuticle scales being removed, and the inner cortex being exposed.

To further assess the extent of incorporation and to compare the artificially incorporated samples to authentic drug containing samples, single hair analysis and drug mapping was proposed. However, due to interferences with PDMS contamination, and PDMS within the hair sample, the use of ToF-SIMS was deemed not suitable for single hair analysis at this time. Future work is needed to resolve the issues.

Chapter 7

Conclusions & future work

7.1 Conclusions

This research outlines the use of alternative methods of extraction as a potential substitute for an 18-hour incubation process used for the analysis of hair. In addition, this thesis presents research towards effect of some cosmetic hair treatments on hair and the drug concentration in hair, and a method of artificially incorporating drugs into hair. Chapter 3 describes a method of extracting drugs from hair using microwave energy. It was demonstrated that by heating the hair in methanol (2 mL) in the microwave at 70 °C that the extraction time can be reduced from 18 hours to 2 hours when compared to heating at 45 °C in a conventional oven. Using the microwave-assisted extraction method, the concentrations of the 12 target analytes assessed were, on average, greater than through using the 18-hour incubation method. Following the extraction, the methanol is collected, evaporated and reconstituted, with no additional clean-up required. However, due to potential contamination of samples from re-using the microwave tubes, new tubes will need to be used for each extraction, which can be expensive, or a more rigorous, reliable cleaning method will need to be employed, to make microwave assisted extraction a viable method.

The use of microwave energy was continued for the study presented in Chapter 4 with an ionic liquid composite (ILC) used in place of methanol for the extraction of drugs. The study presented in Chapter 4 investigates the dissolution of hair in an ILC followed by a liquid-liquid extraction (LLE) to allow for the analysis of drugs. It was demonstrated that although using a urea/choline chloride (2:1 molar) ionic liquid composite could achieve dissolution of hair, this was difficult to accomplish at temperatures low enough to avoid potential drug degradation. Conventional methods of heating require either high temperatures (~150 °C) or long periods of time to disassemble the hair with the urea/choline chloride (2:1 molar) ILC. With the assistance of microwave digestion, the dissolution process takes less time, but the temperature could not be reduced. Using lower set temperatures and lower power outputs can reduce the temperature reached by a few degrees, but very little dissolution occurs. Even with a slight reduction in temperature, loss of drug through thermal degradation was still an issue. Additionally, the extraction and clean-up procedures that followed the digestion were difficult due to the high viscosity of the ILC, resulting in low recoveries. Consequently, the method using the urea/choline chloride ILC and microwave heating was determined to be unsuitable

for drug detection in hair, however, there may be potential for other uses such as detection of heavy metals.

Chapter 5 investigated the effects of the Olaplex® treatment and a bleach treatment on the concentration of drugs in hair and the effect on the overall structure of hair. By treating hair containing artificially incorporated drugs with regular (weekly) shampoo and conditioner, there was little to no change in the detected drug concentrations. In contrast, upon treating the hair with just the Olaplex® treatment (without bleach) the detected drug concentrations were reduced. This reduction was small compared to the effect bleaching has on the drug content. Bleaching has the largest impact on concentration, with a substantial decrease in measured drug concentrations seen after just the in-salon treatments, and a gradual decline following with weekly washes. Using the Olaplex® incorporated into the bleach treatment had a similar effect on the drug content in hair as the stand alone bleach treatment. However, by the final at-home treatments using the Olaplex®, more of the compounds were able to be detected at higher concentrations. Bleaching had the largest impact in regards to structural damage, with the complete removal of cuticle scales and additional damage to the keratin fibres of the cortex for most of the hairs examined. The use of Olaplex® drastically reduced the damage done by bleach. It was not possible, using SEM or ATR-FTIR, to determine whether a hair had been treated using Olaplex rather than another oxidative hair treatment.

Chapter 6 investigated how temperature, solvent ratios, and prior cosmetic treatment affect the artificial incorporation of drugs into hair. Artificial incorporation through soaking hair in a drug mix made up in methanol and DMSO mix produced higher detected concentrations of the target analytes in the hair when incubating at 35 °C for the 7 day soak, and using a higher MeOH:DMSO ratio. The hair bleached prior to artificial incorporation also resulted in higher concentrations in the hair, which supports previous work done. The hair treated with Olaplex® prior to incorporation had the opposite effect and prevented some of the drugs from incorporating, with the exception of benzodiazepines, which showed an increase. Through microscopic analysis, damage to the hair during the drug incorporation process was observed. For a large proportion of the hairs assessed the damage was minimal, with some of the cuticle scales being removed. However, more extensive damage was also observed with numerous cuticle

scales being removed, and the inner cortex being exposed. To further assess the extent of incorporation and to compare the artificially incorporated samples to authentic drug containing samples, single hair analysis and drug mapping through ToF-SIMS analysis was proposed. However, due to interferences with PDMS contamination and PDMS within the hair sample, the use of ToF-SIMS was deemed not suitable for single hair analysis at this time.

7.2 Future Work

7.2.1 MAE with a multi-vessel microwave

Chapter 3 investigated the use of MAE to reduce the time required for the extraction of drugs from hair. Since only a single-vessel microwave was available for use, the number of samples and replicates able to be performed in one day was restricted. Therefore, to ensure the study was carried out in a timely manner only microwave time and temperature were assessed as potential variants. Further investigations using a multi-vessel microwave should ideally be completed to enable assessment of a greater range of microwave time and temperature as well as different microwave power outputs and extraction solvents. Additionally, the intraday precision was not conducted due to limitations with the sample capacity of the microwave, but the use of a multi-vessel microwave will allow for the intraday precision to be assessed. A single-vessel microwave is also not practical if being used for drug screening, or batch analysis in a forensic laboratory, and hence a multi-vessel microwave will need to be used regardless.

7.2.2 Ball-milling prior to extraction

It was also mentioned in Chapter 3 that ball-milling or pulverising the hair prior to MAE instead of manually cutting the hair into small fragments would be beneficial, as previous studies have indicated that this will result in greater extraction, over a shorter time. This could enable the MAE method to be reduced even further than the optimised 2 hours. Ball-milling was intended to be assessed during this research, however preliminary studies using a ball mill were conducted by Forensic Science SA prior to the commencement of this project, and a number of issues arose from this study. In this study, hair (100 mg) was pulverised, then a subsample (20 mg) was collected for analysis. The detected concentration of analyte in hair was less than if the traditional cutting method was used, which contradicts results from previous studies. It was hypothesised

that the analytes were sticking to either the inside of the tube or the ball bearings, or breaking down as a result of extremely high pressures at the small points of contact between balls. It was suggested to resolve the issue of the analytes sticking, that 20 mg of hair could be ball-milled with the extraction solvent simultaneously, followed by the collection and analysis of the solvent. However, during the ball-milling process, the tubes designed to fit the mill would crack, which would result in the solvent leaking. This could also lead to issues with cross-contamination. Further investigations into using ball-milling or pulverisation the hair prior to extraction would be beneficial for this research.

7.2.3 Assessment of different ionic liquid composites and deep eutectics

Chapter 4 investigated the disassembling of hair using the ionic liquid composite (ILC) urea/choline chloride (2:1 molar). This particular ILC was selected based on previous research of colleagues at Flinders University. This was the only ILC assessed, and hence in future work, additional ionic liquids and I ionic liquid composites should be assessed, such as the various other choline chloride mixes, and the 1-allyl-3-methylimidazolium dicyanamide ionic liquid which has previously been used for the dissolution of wool [187].

7.2.4 Use of reducing agents to disassemble the structure of hair

Reducing agents have been used previously for hair analysis to assist in enzymatic digestion. The purpose of the reducing agents is to break up the longitudinal chains of the keratin protein of the hair by reducing the disulphide bonds. These compounds are also quite often used in the first stage of perming hair treatment. The reducing agent most used is dithiothreitol (DTT), with Proteinase K enzyme. A preliminary study was undertaken to assess the potential for reducing agents, DTT and tris(2-carboxyethyl) phosphine hydrochloride (TCEP), to disassemble the structure of hair to a similar extent as the ILC, without the assistance of an enzyme. Solutions of DTT (2.5, 5, 10 and 20 mg/mL) and TCEP (25 mM) and were mixed with hair sample 7 (1.5mL: 20mg) for 5 hours at 50 °C and 80 °C. None of the DTT solutions resulted in any change to the hair, however the 25 mM TCEP solution heated at 80 °C resulted in the hair appearing paler in colour and becoming very brittle. This sample was analysed through transmitted light microscopy (refer to Figure 41) and SEM (refer to Figure 42) to determine what effect the TCEP and heating had on the structure and appearance of the hair. Both techniques clearly indicated that the outer cuticle scales are still intact and undamaged, however the

overall structure of the hair has been distorted. The hair shaft appears to have collapsed in on itself, resulting in the buckling effect.

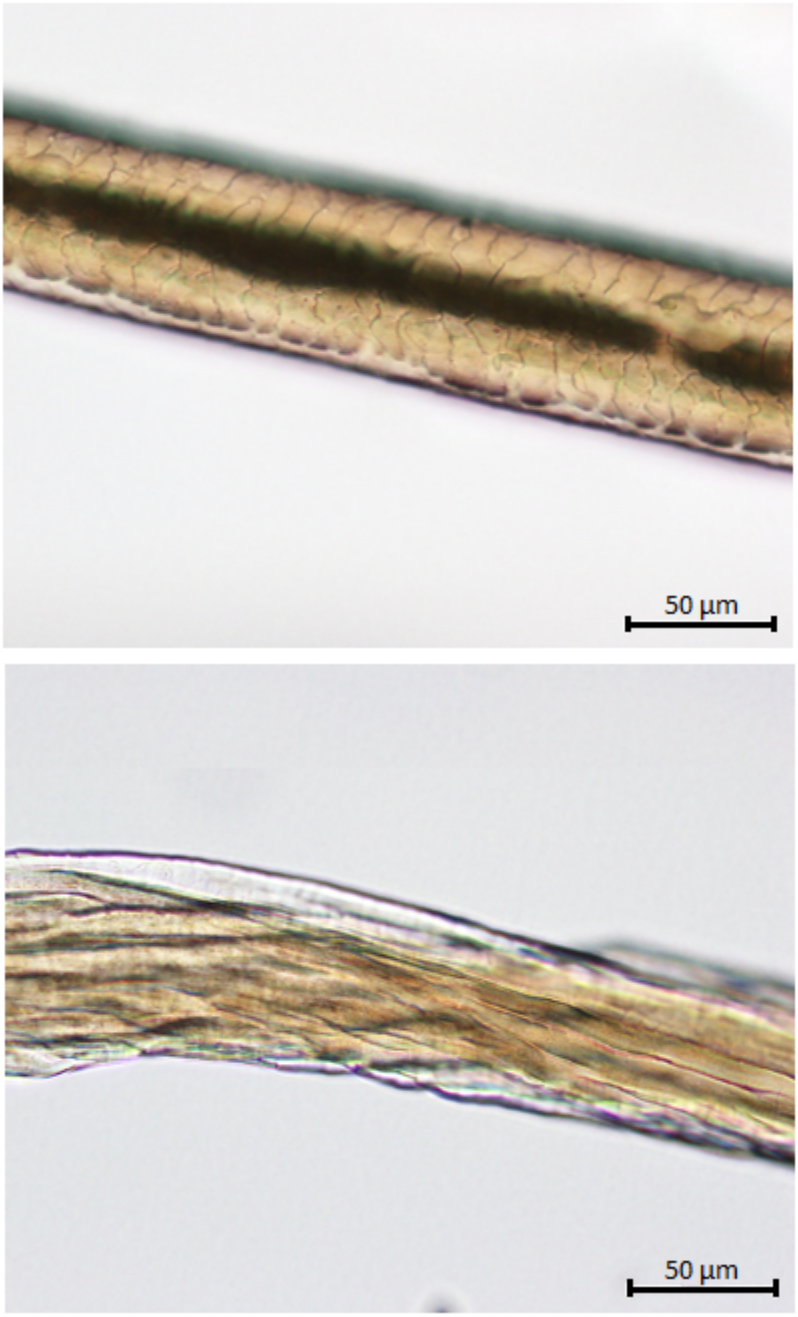


Figure 41: Transmitted light microscopy images of untreated donor hair (top) and hair heated for 5 hours at 80 °C with 25 mM TCEP solution (bottom). Images taken an Olympus CX41 Compound microscope

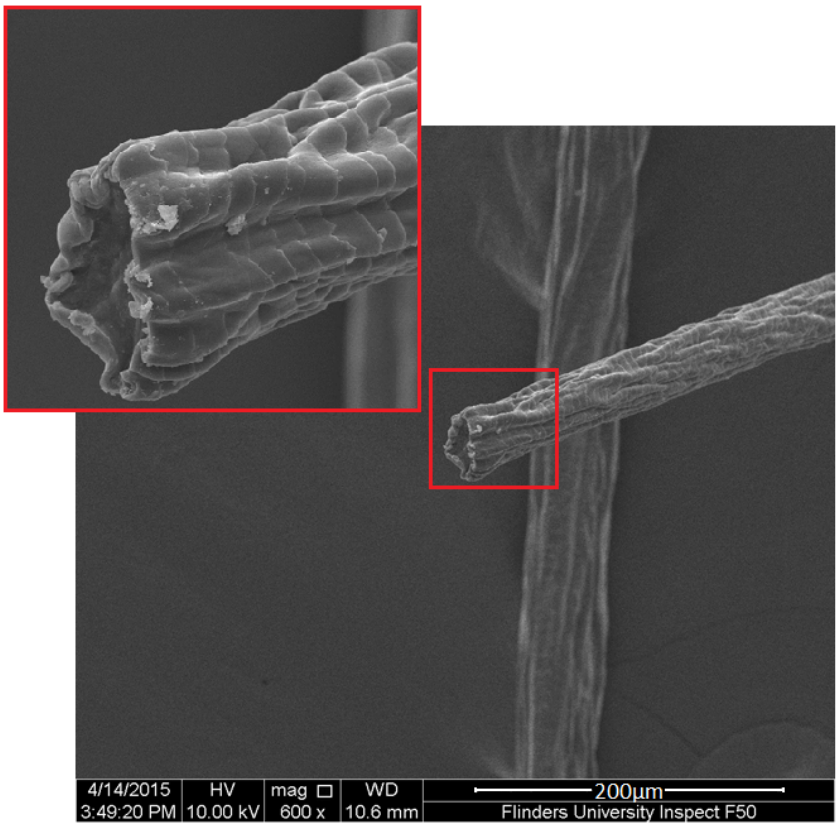


Figure 42: SEM of hair fibres treated with 25 mM TCEP solution for 5 hours and 80 °C. Images taken using Inspect FEI F50 SEM

7.2.5 Investigation into Temazepam after Olaplex® treatment

Chapter 5 investigated the effects of the Olaplex® treatment and a bleach treatment on the concentration of drugs in hair and the effect on the overall structure of hair. While most of the target analytes showed a decline in drug concentration over the number of Olaplex® and wash cycles, temazepam exhibited an increase. Similarly, in Chapter 6, temazepam had higher drug content in the Olaplex® treated hair prior to artificial drug incorporation. It was speculated in Chapter 6 that the Olaplex® and the standard at-home conditioning treatments could be pulling the drug out of the hair, but not removing it, making the methanol extraction easier. Further investigation into why this is occurring is needed. Single hair analysis and drug mapping could help verify why the results for temazepam were different from the other target analytes. It could also determine whether the decrease in drug concentration after the Olaplex® treatment (without bleaching) is a result of the drug being removed during the treatment process or locking

the drug into the hair, making it harder to extract. If it is due to the drug being locked into the hair ball-milling may be beneficial to the extraction process.

7.2.6 Artificial drug incorporation – how effective is it?

Chapter 6 investigated the production of artificially drug incorporated hair standards through a solvent soak. One question that arose from this study was how accurately does the artificially incorporated hair represent an authentic drug containing hair sample, in terms of drug location within the hair? ToF-SIMS was briefly looked at as a way of determining this. However, there was too much interference from PDMS signals. Additional experiments to determine how to reduce the PDMS noise are needed. Different mounting methods will not help if the interfering PDMS is coming from within the hair, rather than contamination, as initially thought. A possible solution may be to use the ToF-SIMS ion beam to sputter away the surface of the hair in order to remove surface siloxanes, which could also confirm whether the siloxane interferences are just on the surface, or throughout the entire shaft of hair. Using an alternative ion gun may enable better resolution of the target analytes, with less interference from the siloxanes.

Appendix

MICROWAVE-ASSISTED EXTRACTION

Table 31: Stability of target analytes after being microwaved (2 hours, 70 °C), showing the percent loss of drug ± 1 SD (where n=3)

	Concentration 1	Concentration 2	Concentration 3
AMP	14.9 ± 4.37	3.74 ± 1.72	5.88 ± 3.01
MDMA	-0.0486 ± 2.22	-2.36 ± 5.43	0.487 ± 4.84
METH	16.9 ± 6.65	2.64 ± 9.96	9.66 ± 4.18
MOR	-22.6 ± 3.53	-11.8 ± 7.75	-6.93 ± 6.62
COD	-22.6 ± 5.59	3.46 ± 5.33	-2.02 ± 6.78
TEM	-6.17 ± 1.91	-7.75 ± 4.02	-3.57 ± 3.50
OXAZ	18.2 ± 2.94	27.6 ± 2.00	25.3 ± 5.31
NOR	-10.9 ± 6.66	-4.71 ± 8.24	-4.15 ± 4.05
DIAZ	-6.52 ± 5.20	2.60 ± 3.69	4.63 ± 4.08
MET	1.49 ± 11.0	21.8 ± 4.67	10.8 ± 0.972
COC	-1.42 ± 0.988	-0.302 ± 2.57	-8.03 ± 14.0
OXY	-12.9 ± 13.5	2.7 ± 3.70	4.08 ± 3.31

Table 32: Stability of target analytes in the final extract (in 0.1 % formic acid in water), showing the percent loss of drug ± 1 SD (where n=3) 1 day and 1 week after the initial injection. Results calculated using only the peak area of the analyte, and not the peak area ratio (where n=3)

		Sample		Neat Standard	
		1 day	1 week	1 day	1 week
AMP	Conc. 1	5.03 ± 5.05	-15.2 ± 12.9	2.45 ± 4.95	-15.7 ± 15.5
	Conc. 2	8.81 ± 5.54	-20.3 ± 12.8	8.52 ± 8.67	-8.05 ± 12.7
	Conc. 3	8.45 ± 2.79	-17.7 ± 13.3	-0.36 ± 1.98	-17.8 ± 11.6
MDMA	Conc. 1	0.57 ± 1.68	-8.52 ± 8.07	-1.81 ± 2.97	-11.0 ± 10.4
	Conc. 2	-0.08 ± 2.65	-8.41 ± 8.81	-2.35 ± 7.76	-11.2 ± 12.9
	Conc. 3	-0.83 ± 4.69	-12.0 ± 9.87	-2.30 ± 3.22	-10.4 ± 9.79
METH	Conc. 1	1.34 ± 5.14	-12.9 ± 14.3	-2.14 ± 13.0	-16.4 ± 18.2
	Conc. 2	2.95 ± 4.48	-13.0 ± 15.4	-4.96 ± 9.33	-14.8 ± 18.1
	Conc. 3	-1.11 ± 6.09	-17.2 ± 14.0	-3.36 ± 6.25	-19.4 ± 14.7
MOR	Conc. 1	11.4 ± 8.87	-17.1 ± 5.96	5.08 ± 2.30	-2.75 ± 6.59
	Conc. 2	12.7 ± 7.10	-8.97 ± 3.58	0.63 ± 3.12	-9.13 ± 8.81
	Conc. 3	9.64 ± 8.88	-8.98 ± 3.10	1.85 ± 2.41	-8.48 ± 9.29
COD	Conc. 1	-3.78 ± 4.20	-22.5 ± 9.89	3.45 ± 3.89	1.29 ± 4.02
	Conc. 2	-0.82 ± 8.08	-12.5 ± 4.87	3.67 ± 8.75	-8.05 ± 7.67
	Conc. 3	6.29 ± 1.96	-5.37 ± 4.24	-0.03 ± 1.44	-4.34 ± 2.79
TEM	Conc. 1	19.6 ± 7.29	28.9 ± 10.6	10.5 ± 7.51	25.2 ± 11.6
	Conc. 2	19.3 ± 7.95	39.0 ± 13.1	11.8 ± 4.88	24.7 ± 9.76
	Conc. 3	17.4 ± 5.78	28.9 ± 7.25	14.1 ± 4.59	25.7 ± 8.53
OXAZ	Conc. 1	0.70 ± 15.2	-6.20 ± 4.61	-2.14 ± 12.2	0.650 ± 13.8
	Conc. 2	6.32 ± 10.8	11.3 ± 14.7	5.91 ± 5.85	2.45 ± 10.4
	Conc. 3	3.62 ± 6.05	6.17 ± 7.94	2.65 ± 5.12	1.50 ± 10.5
NOR	Conc. 1	36.3 ± 9.56	57.8 ± 3.84	25.5 ± 9.53	42.8 ± 10.7
	Conc. 2	30.8 ± 6.57	53.2 ± 7.58	29.7 ± 5.55	45.8 ± 8.97
	Conc. 3	33.3 ± 3.39	54.8 ± 1.27	29.4 ± 1.27	45.9 ± 8.01
DIAZ	Conc. 1	0.91 ± 2.83	-15.7 ± 37.1	-6.35 ± 11.5	6.65 ± 1.64
	Conc. 2	9.02 ± 1.08	6.36 ± 6.06	6.20 ± 3.75	9.02 ± 1.97
	Conc. 3	1.17 ± 3.17	1.64 ± 2.97	7.09 ± 0.49	7.42 ± 0.89
MET	Conc. 1	15.0 ± 9.46	14.5 ± 5.29	0.06 ± 17.0	0.541 ± 16.9
	Conc. 2	1.43 ± 6.45	3.61 ± 8.48	0.11 ± 14.4	0.765 ± 13.0
	Conc. 3	7.91 ± 3.52	23.4 ± 10.3	3.39 ± 10.8	4.86 ± 11.8
COC	Conc. 1	2.27 ± 5.05	-4.05 ± 6.86	-7.27 ± 10.4	-9.93 ± 10.9
	Conc. 2	2.61 ± 7.14	-2.93 ± 4.26	-8.01 ± 11.4	-11.4 ± 15.8
	Conc. 3	3.36 ± 2.02	-3.75 ± 6.43	-6.57 ± 5.89	-10.9 ± 10.0
OXY	Conc. 1	3.71 ± 14.6	3.93 ± 2.36	2.81 ± 2.28	1.98 ± 1.91
	Conc. 2	6.19 ± 6.63	-3.85 ± 5.27	2.28 ± 8.51	0.452 ± 2.38
	Conc. 3	4.73 ± 2.39	-1.65 ± 6.69	1.88 ± 1.86	0.202 ± 2.82

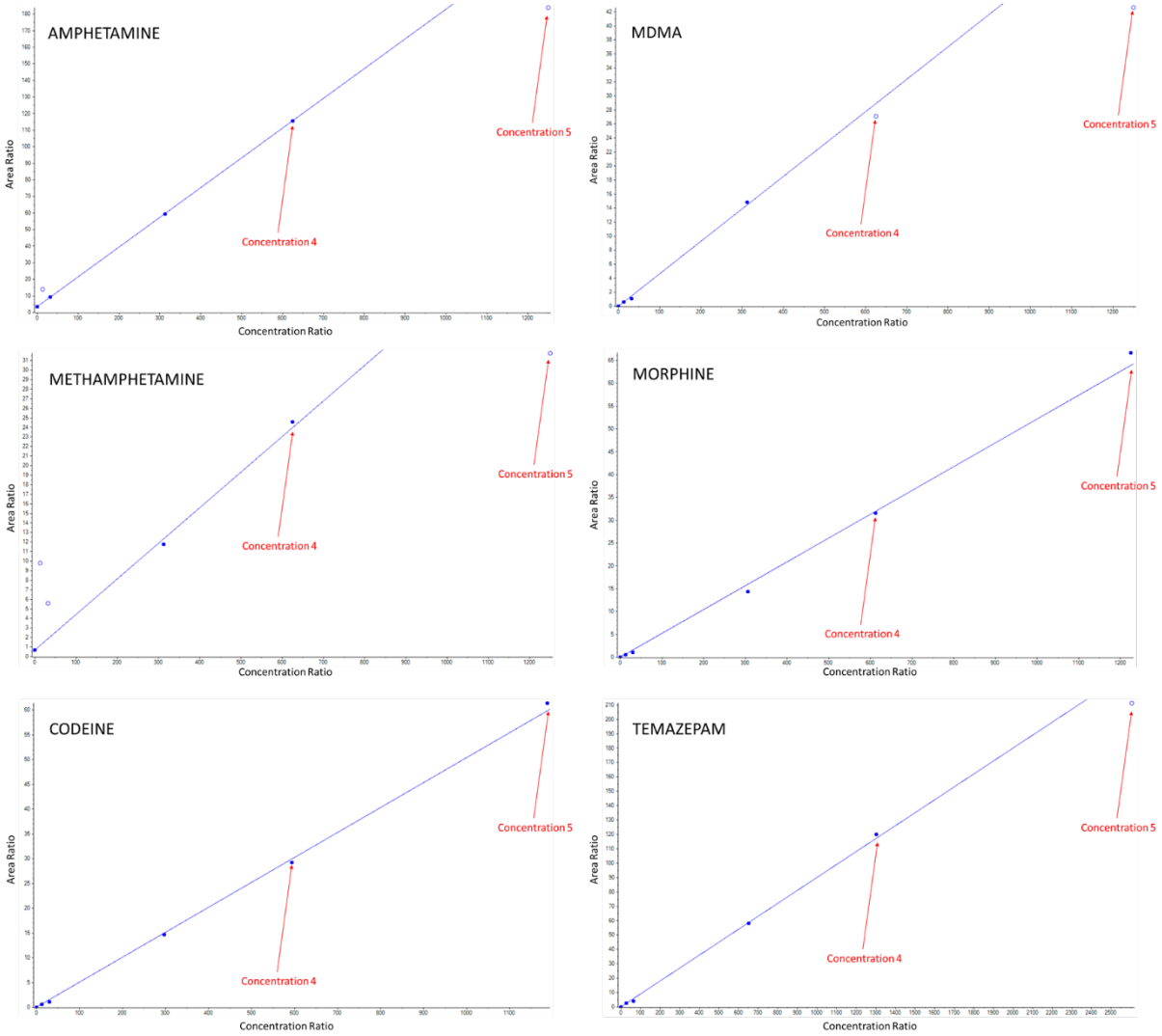


Figure 43: Exemplar calibration curves for each target analyte (AMP, MDMA, METH, MOR, COD and TEM) at the 5 concentrations listed in Table 11, page 49.

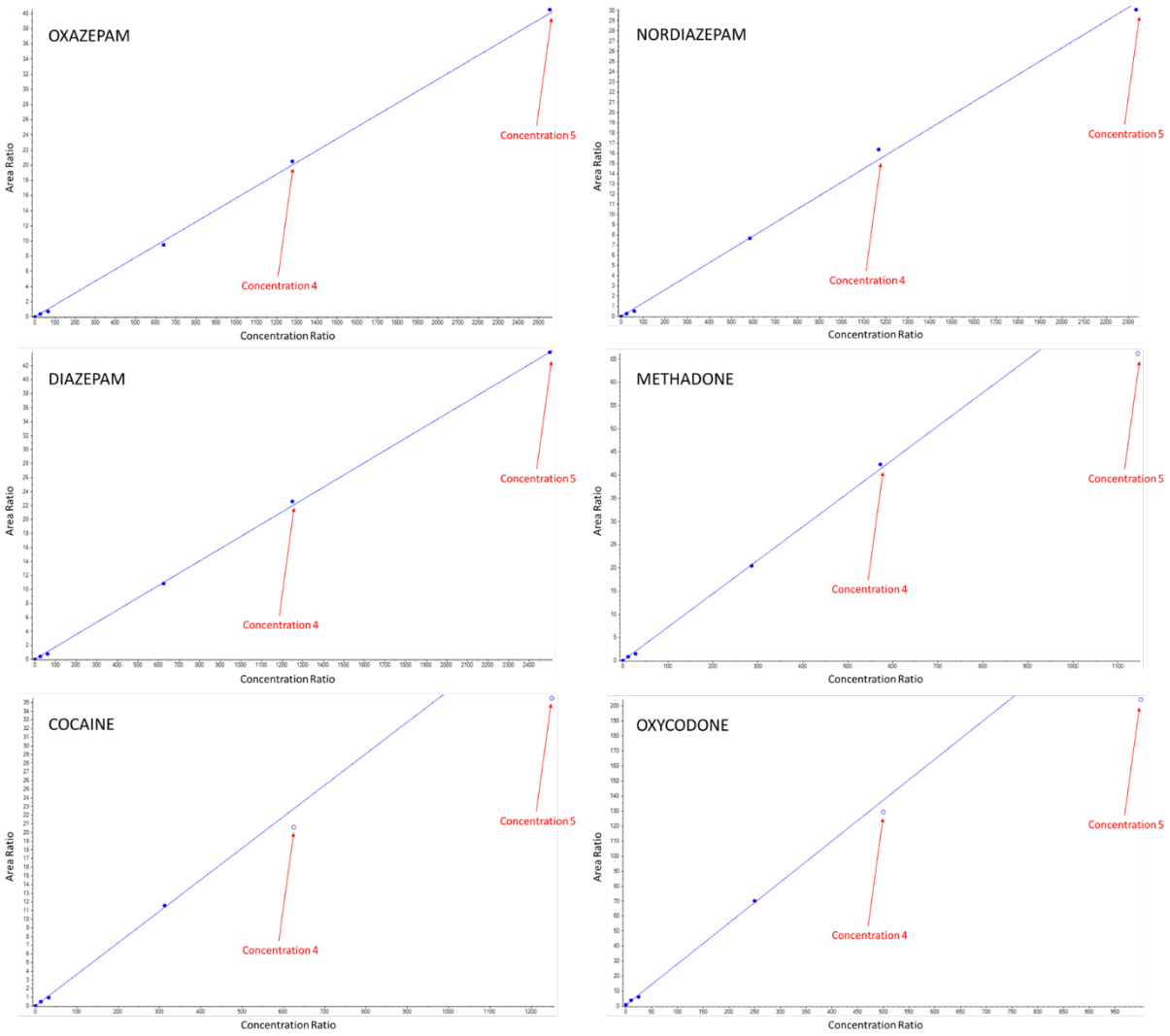


Figure 44: Exemplar calibration curves for each target analyte (OXAZ, NOR, DIAZ, MET, COC and OXY) at the 5 concentrations listed in Table 11, page 49

THE EFFECT OF THE OLAPLEX® TREATMENT ON DRUG DETECTION AND THE OVERALL STRUCTURE OF HAIR

Table 33: Calculated concentrations (pg/mg ± 1 SD) of drugs in artificially incorporated hair, before and after weekly shampoo and conditioning (where n=3)

	Spiked, NO Olaplex®			
	Initial	1 Wash	5 Washes	9 Washes
AMP	511 ± 53.3	482 ± 25.5	565 ± 122	335 ± 40.6
MDMA	396 ± 20.7	446 ± 15.0	351 ± 16.0	292 ± 23.5
METH	381 ± 8.69	354 ± 25.5	277 ± 6.34	184 ± 14.4
MOR	121 ± 8.87	145 ± 8.48	110 ± 5.66	91.4 ± 4.82
COD	161 ± 8.83	175 ± 7.41	135 ± 5.86	112 ± 9.40
TEM	159 ± 16.6	190 ± 8.36	326 ± 48.5	283 ± 12.7
OXAZ	166 ± 10.2	186 ± 3.15	177 ± 11.6	175 ± 4.61
NOR	56.9 ± 3.82	43.9 ± 3.39	58.7 ± 3.39	42.6 ± 5.75
DIAZ	55.1 ± 3.25	45.0 ± 1.09	39.7 ± 2.42	32.9 ± 4.51
MET	104 ± 3.51	136 ± 10.4	149 ± 17.2	134 ± 6.23
COC	51.8 ± 2.15	47.9 ± 2.70	38.1 ± 2.49	31.9 ± 2.39
OXY	75.5 ± 7.63	89.2 ± 9.57	69.4 ± 4.83	56.7 ± 3.60

Table 34: Calculated concentrations (pg/mg ± 1 SD) of drugs in artificially incorporated hair, before and after the in Olaplex® No. 1 and No. 2 treatments (in-salon) and weekly Olaplex® No.3 and washes (where n=3)

	Spiked, FULL Olaplex®				
	Initial	In-salon	1 Wash	5 Washes	9 Washes
AMP	607 ± 46.4	316 ± 4.00	337 ± 9.71	185 ± 29.8	72.4 ± 2.00
MDMA	448 ± 27.5	271 ± 13.4	306 ± 11.1	186 ± 22.1	121 ± 2.13
METH	415 ± 12.1	188 ± 15.7	158 ± 9.87	88.1 ± 12.1	42.4 ± 2.56
MOR	144 ± 11.0	83.8 ± 0.835	112 ± 1.25	69.6 ± 7.08	62.1 ± 3.62
COD	183 ± 13.3	96.6 ± 7.97	104 ± 7.30	77.8 ± 9.32	68.2 ± 2.63
TEM	143 ± 16.2	136 ± 5.91	140 ± 3.50	238 ± 25.8	205 ± 8.35
OXAZ	175 ± 12.6	184 ± 3.70	158 ± 12.9	198 ± 8.45	164 ± 5.92
NOR	46.3 ± 1.94	52.8 ± 3.60	26.7 ± 3.44	54.8 ± 7.75	36.0 ± 4.39
DIAZ	40.9 ± 3.97	35.8 ± 1.36	<LOQ	34.3 ± 6.39	<LOQ
MET	104 ± 3.20	132 ± 0.752	159 ± 10.1	144 ± 18.2	119 ± 5.77
COC	50.1 ± 1.64	31.0 ± 1.27	27.1 ± 0.544	26.3 ± 189	21.1 ± 1.06
OXY	85.0 ± 9.24	57.6 ± 4.77	87.9 ± 1.63	55.5 ± 8.43	50.3 ± 0.309

Table 35: Calculated concentrations (pg/mg ± 1 SD) of drugs in artificially incorporated hair, before and after bleaching and weekly shampoo and conditioning (where n=3)

	Spiked, bleached, NO Olaplex®				
	Initial	In-salon	1 Wash	5 Washes	9 Washes
AMP	1060 ± 410	157 ± 5.02	98.5 ± 6.61	89.9 ± 57.2	13.8 ± 1.70
MDMA	728 ± 241	89.2 ± 2.14	73.5 ± 7.09	29.1 ± 1.20	<LOQ
METH	724 ± 277	80.6 ± 2.59	54.1 ± 3.51	19.7 ± 0.509	<LOQ
MOR	232 ± 80.0	<LOQ	<LOQ	<LOQ	
COD	293 ± 104	<LOQ	<LOQ	<LOQ	
TEM	175 ± 11.5	47.6 ± 1.28	56.3 ± 2.41	94.1 ± 5.46	87.7 ± 2.63
OXAZ	218 ± 24.8	65.7 ± 0.061	60.3 ± 3.24	66.1 ± 3.07	62.6 ± 4.64
NOR	59.0 ± 5.31	36.7 ± 1.31	31.1 ± 3.38	31.9 ± 1.56	24.0 ± 1.80
DIAZ	50.8 ± 3.82	<LOQ	<LOQ	<LOQ	<LOQ
MET	117 ± 26.7	43.2 ± 2.47	31.6 ± 4.09	29.0 ± 0.871	<LOQ
COC	89.9 ± 31.4	19.1 ± 0.862	13.5 ± 0.826	<LOQ	<LOQ
OXY	145 ± 55.3	10.0 ± 1.34	<LOQ	<LOQ	

Table 36: Calculated concentrations (pg/mg ± 1 SD) of drugs in artificially incorporated hair, before and after Olaplex® No. 1 and No. 2 treatments incorporated into bleaching and weekly Olaplex® No.3 and washes (where n=3)

	Spiked, bleached, FULL Olaplex®				
	Initial	In-salon	1 Wash	5 Washes	9 Washes
AMP	1202 ± 442	186 ± 12.1	132 ± 4.91	59.1 ± 7.59	15.0 ± 1.11
MDMA	840 ± 274	94.9 ± 2.64	75.6 ± 2.72	33.6 ± 0.765	14.0 ± 0.149
METH	923 ± 353	79.9 ± 2.52	53.8 ± 5.06	16.0 ± 0.296	<LOQ
MOR	257 ± 83.3	<LOQ	<LOQ	<LOQ	<LOQ
COD	335 ± 124	32.9 ± 8.50	22.6 ± 5.44	<LOQ	<LOQ
TEM	233 ± 31.1	59.1 ± 1.47	74.8 ± 5.79	101 ± 18.0	101 ± 21.1
OXAZ	258 ± 32.3	83.9 ± 7.85	72.0 ± 5.57	92.5 ± 14.9	77.4 ± 20.9
NOR	81.2 ± 13.5	35.8 ± 0.562	31.7 ± 3.22	32.7 ± 1.87	<LOQ
DIAZ	81.9 ± 16.7	26.7 ± 0.728	<LOQ	<LOQ	<LOQ
MET	166 ± 3.81	37.5 ± 2.92	37.8 ± 2.46	38.2 ± 4.14	40.0 ± 2.92
COC	115 ± 44.5	18.5 ± 0.194	14.1 ± 0.822	<LOQ	<LOQ
OXY	169 ± 64.9	28.7 ± 6.00	71.8 ± 32.2	28.4 ± 2.62	<LOQ

Table 37: Calculated concentrations (pg/mg ± 1 SD) of drugs un-spiked hair, before and after weekly shampoo and conditioning (where n=3)

	Blank, Shampoo & Conditioner			
	Initial	1 Wash	5 Washes	9 Washes
AMP	<LOQ		<LOQ	<LOQ
MDMA				
METH	<LOQ	<LOQ	<LOQ	
MOR				
COD				
TEM				
OXAZ				
NOR				
DIAZ				
MET				
COC				
OXY				

Table 38: Calculated concentrations (pg/mg ± 1 SD) of drugs in un-spiked hair, before and after the in Olaplex® No. 1 and No. 2 treatments (in-salon) and weekly Olaplex® No.3 and washes (where n=3)

	Blank, FULL Olaplex®				
	Initial	In-salon	1 Wash	5 Washes	9 Washes
AMP	16.2 ± 1.99	<LOQ	<LOQ		<LOQ
MDMA	<LOQ				
METH	14.7 ± 1.76	<LOQ	<LOQ		
MOR	<LOQ				
COD					
TEM					
OXAZ					
NOR					
DIAZ					
MET					
COC					
OXY	<LOQ				

Table 39: Calculated concentrations (pg/mg) from the wash of one replicate of each sample (where the wash involves 2 mL methanol swirled with the 20 mg of hair for 30 seconds)

		AMP	MDMA	METH	MOR	COD	TEM	OXAZ	NOR	DIAZ	MET	COC	OXY
Spiked, NO Olaplex®	Initial	33.0	15.2	13.4	<LOQ				<LOQ		<LOQ		
	1 Wash	18.1	<LOQ	<LOQ							<LOQ		<LOQ
	5 Washes	23.2	<LOQ	<LOQ	<LOQ	<LOQ			<LOQ		<LOQ		<LOQ
	9 Washes	23.8	14.8	<LOQ	<LOQ	<LOQ					<LOQ		<LOQ
Spiked, FULL Olaplex®	Initial	20.6	<LOQ	<LOQ							<LOQ		<LOQ
	In-salon	45.2	<LOQ	<LOQ									
	H/T wk. 1	<LOQ	<LOQ	<LOQ							<LOQ		
	H/T wk. 5	35.7	16.4	<LOQ		<LOQ					<LOQ		
	H/T wk. 9	<LOQ	<LOQ	<LOQ		<LOQ					<LOQ		
Spiked, bleached, FULL Olaplex®	Initial	54.3	33.4	34.5	18.2	16.6			<LOQ		<LOQ	<LOQ	12.0
	In-salon	<LOQ	<LOQ	<LOQ									
	H/T wk. 1	<LOQ	<LOQ										
	H/T wk. 5	25.4											
	H/T wk. 9										<LOQ		
Spiked, bleached, NO Olaplex®	Initial	758	25.8	26.7	15.6	15.3					<LOQ	<LOQ	<LOQ
	In-salon	<LOQ	<LOQ										
	H/T wk. 1	<LOQ											
	H/T wk. 5	<LOQ											
	H/T wk. 9												

Table 40: Calculated concentrations (pg/mg) from the wash of one replicate of each sample (where the wash involves 2 mL methanol swirled with the 20 mg of hair for 30 seconds)

		AMP	MDMA	METH	MOR	COD	TEM	OXAZ	NOR	DIAZ	MET	COC	OXY
Blank, Shampoo & Conditioner	Initial	30.6		<LOQ									
	H/T wk. 1	15.2											
	H/T wk. 5	<LOQ											
	H/T wk. 9	<LOQ											
Blank, FULL Olaplex®	Initial	15.5											
	In-salon	<LOQ											
	H/T wk. 1	<LOQ											
	H/T wk. 5												
	H/T wk. 9												

Figure 45: Hair locks treated with Olaplex® (left), bleach (middle), bleach + Olaplex® (right) after the in-salon treatments (top) and after an additional 5 at-home treatments (bottom)

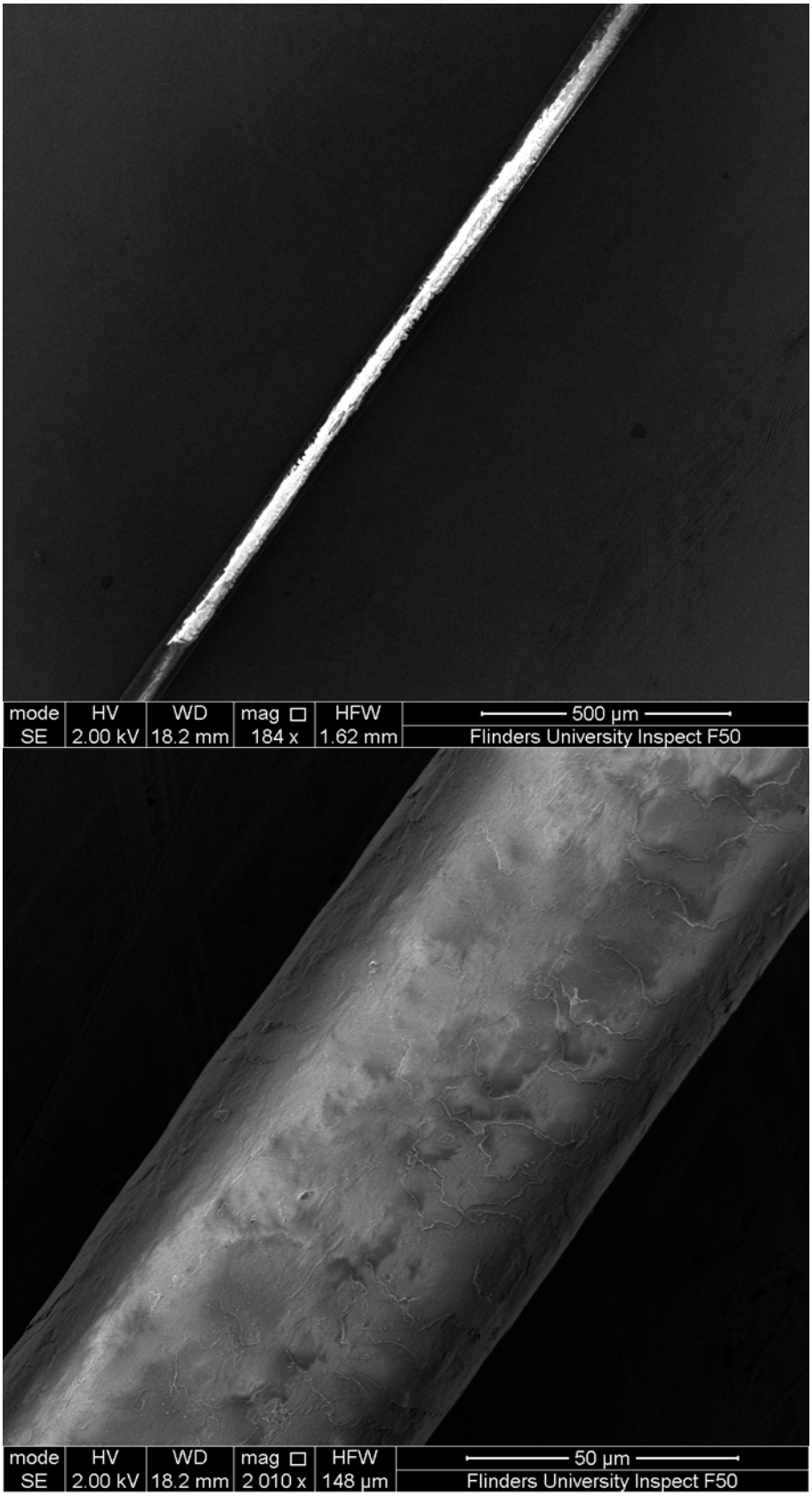


Figure 46: SEM images of hair that has been damaged by the 10 kV ion beam. Images taken using 2 kV ion beam on the Inspect FEI F50 SEM

ARTIFICIAL INCORPORATION OF DRUGS INTO HAIR

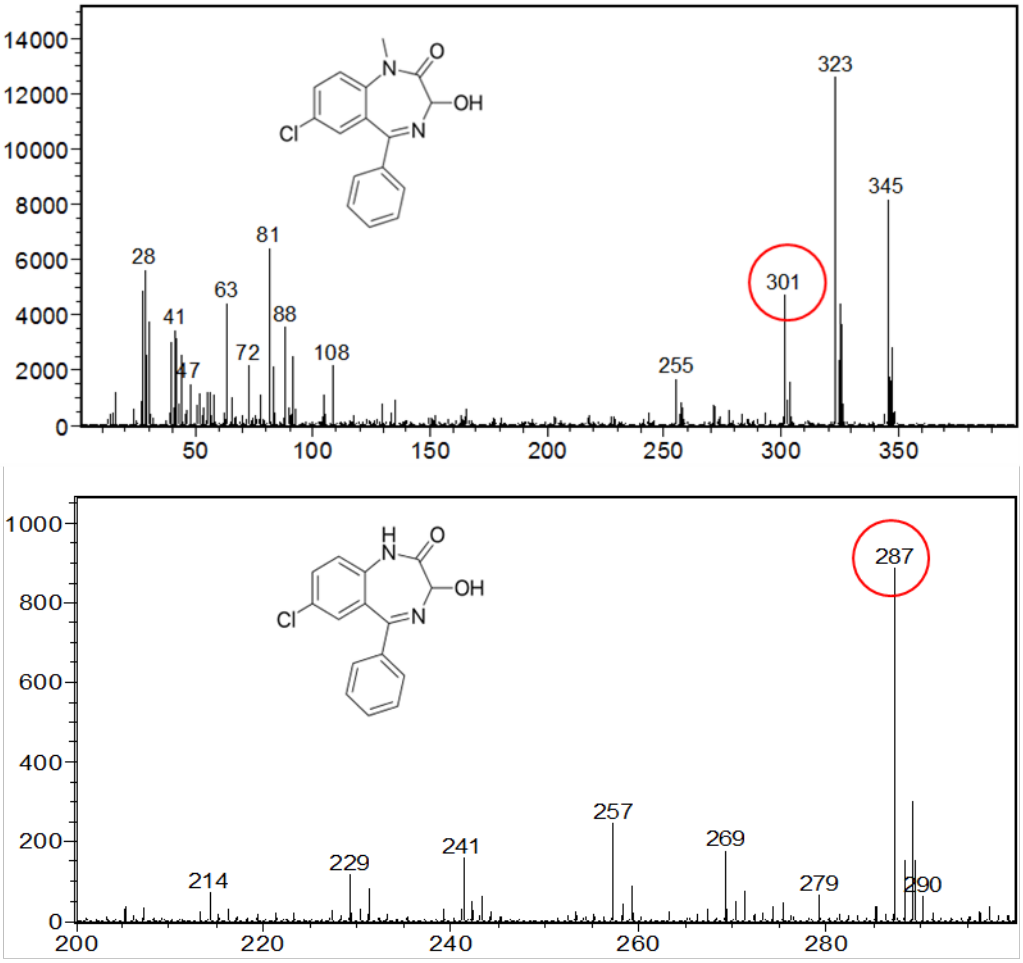


Figure 47: ToF-SIMS reference spectra for temazepam (MW= 300.7 g/mol), where the molecular ion appears at ~301 m/z (top), and oxazepam (MW= 286.71 g/mol), where the molecular ion appears at ~287 m/z (bottom)

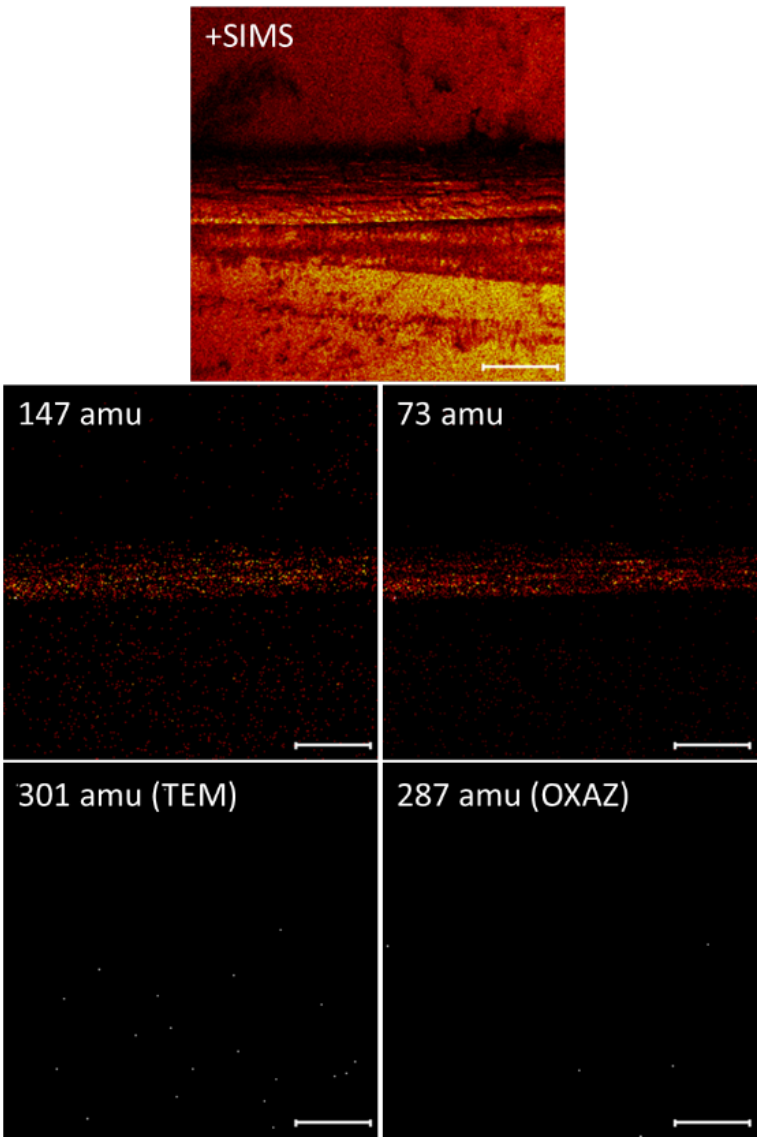


Figure 48: ToF-SIMS images for the authentic drug containing hair (scraped to reveal inner cortex) of total ion count (top), 147 and 73 ions indicating presence of PDMS (middle, left and right), 301 ion indicating temazepam (bottom, left) and 287 ion indicating oxazepam (bottom, right)

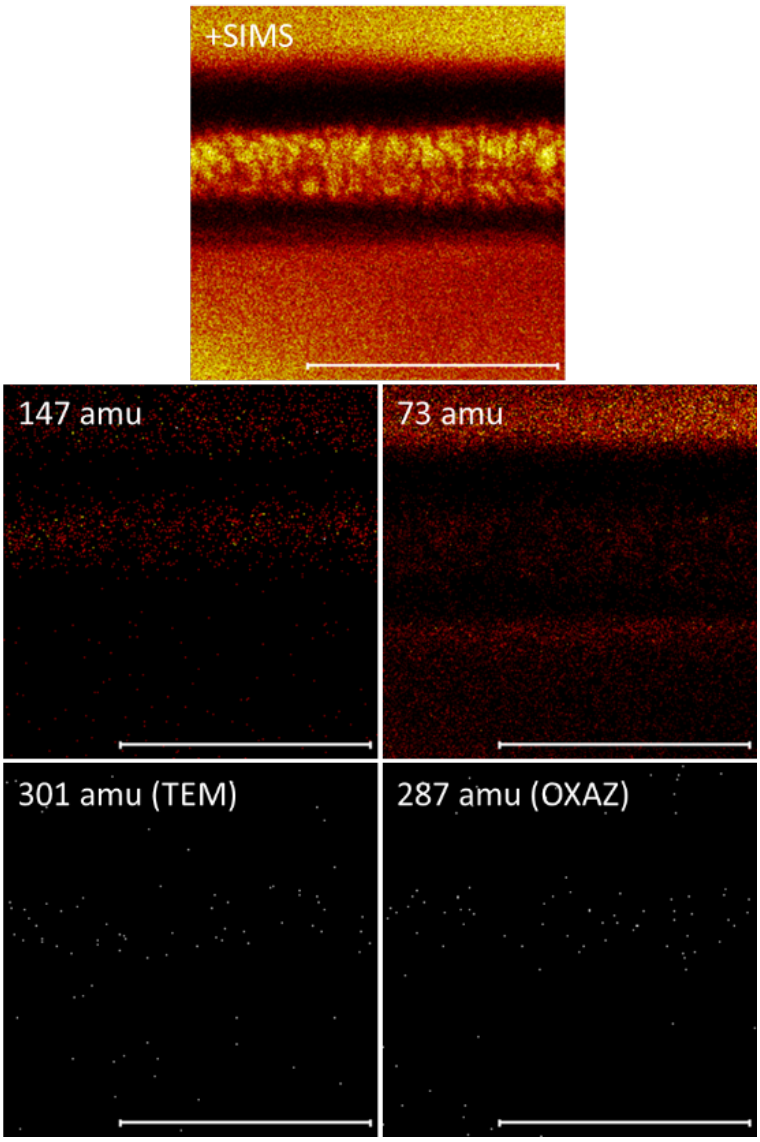


Figure 49: ToF-SIMS images for the authentic drug containing hair (not scraped) of total ion count (top), 147 and 73 ions indicating presence of PDMS (middle, left and right), 301 ion indicating temazepam (bottom, left) and 287 ion indicating oxazepam (bottom, right)

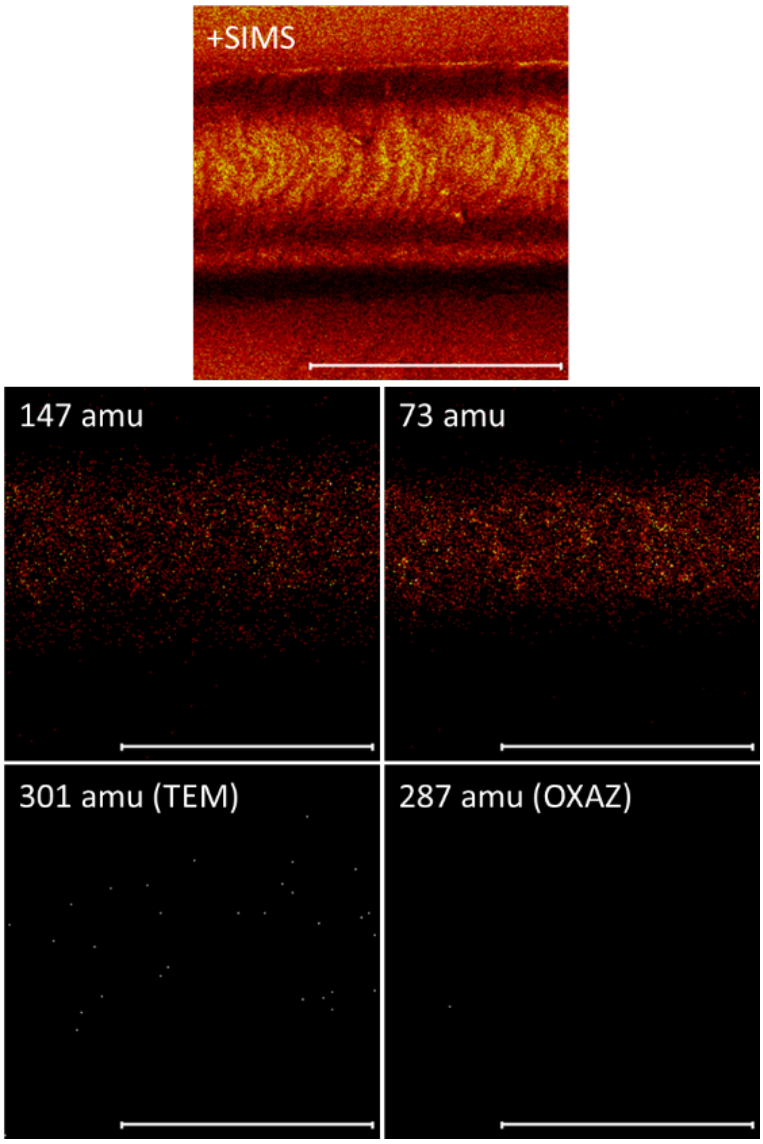


Figure 50: ToF-SIMS images for the blank (drug free) sample (not scraped) of total ion count (top), 147 and 73 ions indicating presence of PDMS (middle, left and right), 301 ion indicating temazepam (bottom, left) and 287 ion indicating oxazepam (bottom, right)

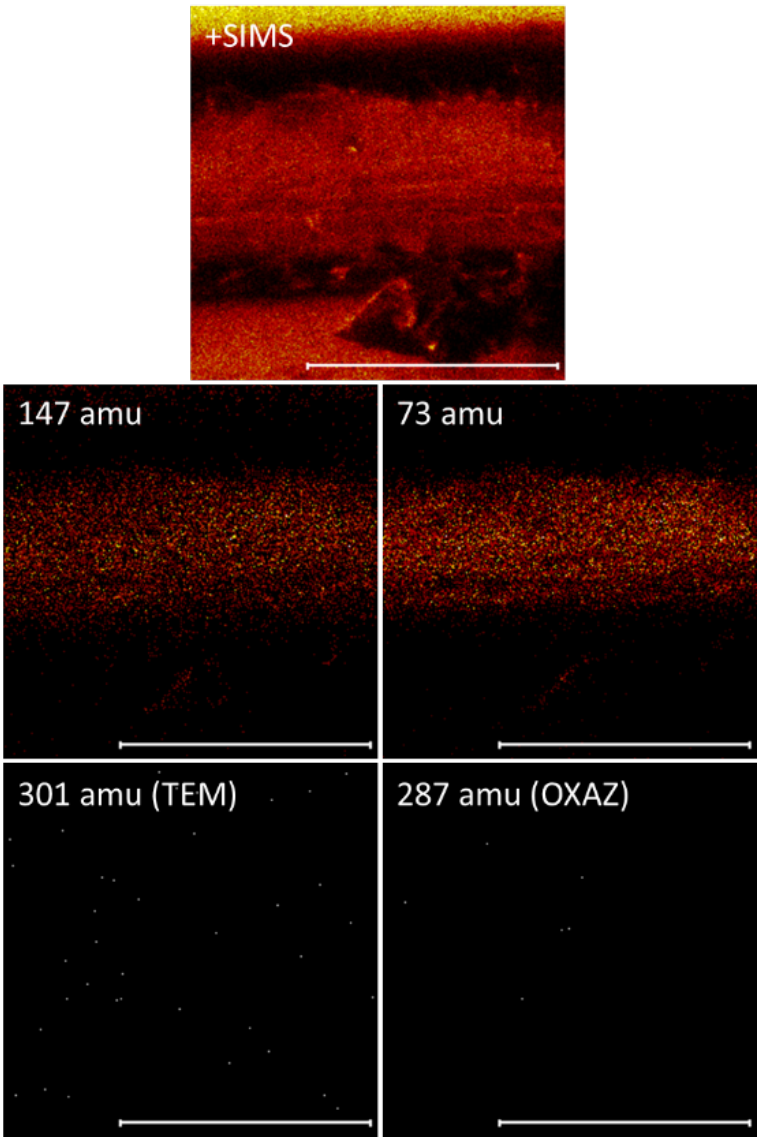


Figure 51: ToF-SIMS images for the blank (drug free) sample (not scraped) of total ion count (top), 147 and 73 ions indicating presence of PDMS (middle, left and right), 301 ion indicating temazepam (bottom, left) and 287 ion indicating oxazepam (bottom, right)

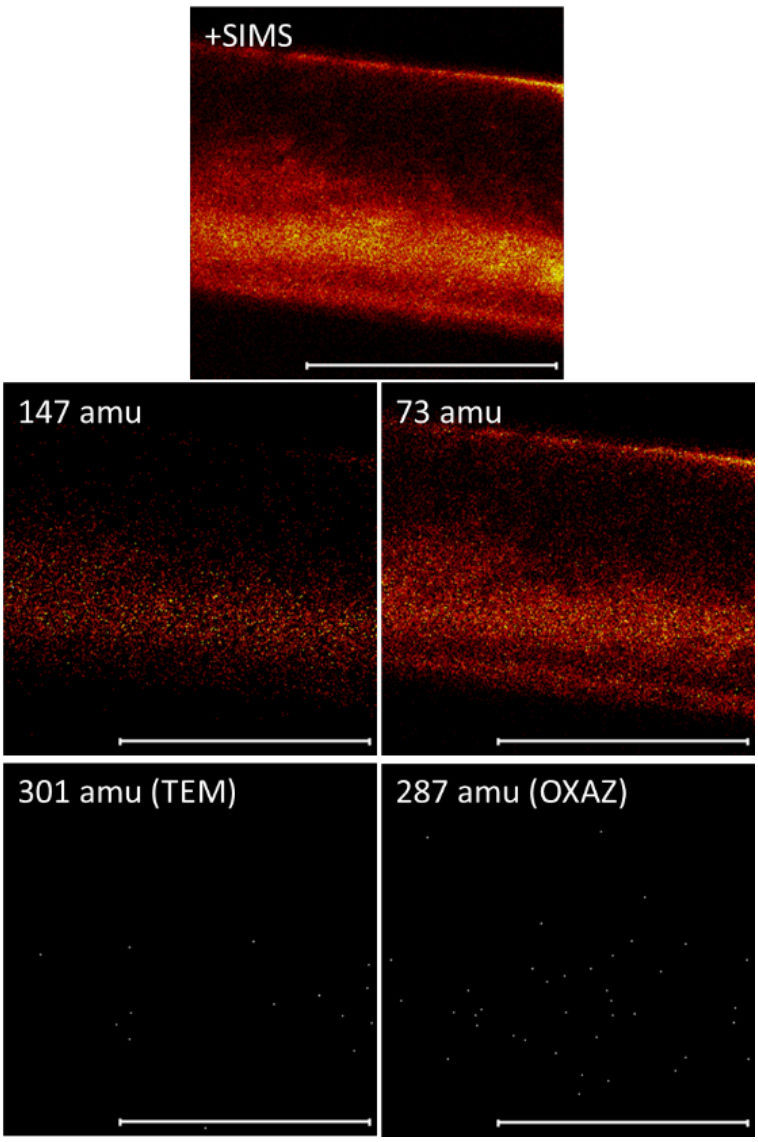


Figure 52: ToF-SIMS images for the artificially drug incorporated sample (not scraped) of total ion count (top), 147 and 73 ions indicating presence of PDMS (middle, left and right), 301 ion indicating temazepam (bottom, left) and 287 ion indicating oxazepam (bottom, right)

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