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Development of a method for the screening and quantification of methamphetamine, and its major metabolite amphetamine, in hair using Liquid Chromatography-Tandem Mass Spectrometry

by

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SUBMITTED TO THE UNIVERSITY OF CAPE TOWN
In partial fulfilment of the requirements for the degree

MPhil (Biomedical Forensic Science)

Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN

March 2015

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Declaration

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Acknowledgements

This study has taken me on quite a journey and there are a few important people who have supported me throughout and to whom I would like to offer my deepest thanks:

Alicia, without you this study would not have been possible. I will always be grateful for the time that you invested in me and for the many hours you sacrificed to teach me. I have learnt so much from you and am inspired by the passionate approach that you have towards your work. Working with you, and learning from you, has been an enriching experience, and the many conversations and laughs that we shared will not be forgotten. Thank-you for everything you have done to help me in completing this journey.

Pete, I am thankful for the continuous support and guidance that you have given me. Your approachable manner and constant willingness to help made this journey much easier. I am most grateful to you for believing in me and for the encouragement that you provided when I needed it most.

Janke, my lab partner, thank you for your optimism and for never failing to make the best out of every situation. I will always be grateful for your friendship and that we got to share in this journey together.

Lastly I would like to thank **Taahira**, from the Writing Centre. My writing is better because of you and I appreciate the time that you have spent helping me improve sections of my thesis.

Abstract

Hair has, over recent years, become widely recognised as an alternate or complementary matrix to blood and urine for drug analysis. Hair analysis offers a wider detection window after drug exposure than blood or urine testing and can provide a long-term history of an individuals drug use. There are several practical applications of hair analysis for drugs including workplace drug testing, doping control, driving licence re-granting, drug-related deaths and drug-facilitated crimes. As a result hair analysis is currently being performed within various toxicological fields in laboratories around the world. However, before the start of this study no hair analysis for drugs was being performed in South Africa. Therefore, the main aim of this study, as stated in Chapter 1, was to develop a method for the detection and quantification of drugs of abuse, specifically methamphetamine and amphetamine, in hair using High Performance Liquid Chromatography coupled to Mass Spectrometry.

Numerous steps are involved in hair analysis for drugs that include washing hair samples to remove external contamination, extracting drugs from within the hair matrix and analysis of the extract using different analytical techniques. A great deal of literature has been published describing various methodologies that have been developed for hair analysis. An overview of a few of these different methodologies is described in Chapter 2. Research also emphasises the limitations of hair analysis and as such the challenges associated with the accurate interpretation of hair analysis results. Limitations concerning external contamination, bias due to race and hair colour, and the effect of cosmetic treatments are also discussed in Chapter 2.

As per the aim and objectives of the study, a method for the detection and quantification of methamphetamine and amphetamine in hair was developed, and is presented in Chapter 3. Drug-free hair spiked with reference standard was used to establish calibration curves. The method developed was verified by testing amphetamine positive samples of hair collected from drug users. Additionally, this method was used to detect and quantify methamphetamine and amphetamine in samples of hair collected during autopsies of suspected drug-related deaths. The results of the study show that the method developed allowed for the detection and quantification of both amphetamine and methamphetamine in hair. This method was not validated, and future research should involve the expansion and validation of the method performed.

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Abbreviations

GC-MS/MS	gas chromatography - tandem mass spectrometry
HCl	hydrochloric acid
HPLC-MS/MS	high performance liquid chromatography – tandem mass spectrometry
LC-MS/MS	liquid chromatography – tandem mass spectrometry
LLOQ	lower limit of quantification
MDA	methylenedioxyamphetamine
MDEA	methylenedioxyethylamphetamine
MDMA	methylenedioxymethamphetamine
MRM	multiple reaction monitoring
NaOH	sodium hydroxide
QC	quality control
Rt	retention time (measured in minutes)
SDS	sodium dodecyl sulphate
SoHT	Society of Hair Testing
UHPLC-MS/MS	ultra high performance liquid chromatography – tandem mass spectrometry

Units of measurement

cps	counts per second
g	grams
$m.s^{-1}$	meters per second
µm	micrometers
mg	milligrams
mg/L	milligrams per litre
mm	millimetres
mM	millimolar
ng/mg	nanograms per milligram
pg/mg	picograms per milligram
rpm	revolutions per minute
V	volts

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Chapter 1: Research Proposal

1. Introduction

The interest in and recognition of hair analysis for drugs has increased steadily over the last two decades with regards to its use in various toxicological fields. Hair analysis for drugs has many different applications including criminal investigations involving drug-related deaths and drug-facilitated crimes, and monitoring the misuse of drugs, for example in workplace drug testing and drug treatment programs (Cooper, Kronstrand & Kintz, 2012).

Drug analysis is usually performed using biological samples such as blood or urine, however, more recently, there are many advantages promoting the inclusion of hair samples in drug analysis. The main advantage of analysing hair for drugs is that hair has a much wider window of drug detection (months to years) compared to blood (hours to days) or urine (days to weeks) (Hegstad et al., 2008). Other advantages of hair analysis are collection of hair samples is a non-invasive process, samples are easily stored and transported, hair does not decompose like other bodily fluids, and through segmental analysis hair can provide a historical profile of an individuals' drug history (Cooper, Kronstrand & Kintz, 2012).

Whilst hair samples can include beard, arm, leg or pubic hair, head hair collected from the area at the back of the head is generally preferable as the growth rate of hair in this area is more constant (Kintz, 2004). It is generally understood that drugs can enter into the hair either by adsorption from the external environment (aerosols or smoke) or by incorporation into the growing hair shaft from blood supplying the hair follicle. Other mechanisms of drug incorporation in hair include diffusion from sweat or sebum secretions (Henderson, 1993).

The application of hair analysis for drugs in post-mortem toxicology is wide and is particularly useful when the history of drug use may be difficult to obtain. In most cases, hair analysis performed complementary to blood analysis, has the potential to help establish the role of drugs with respect to the possible cause of death (Cooper, Kronstrand & Kintz, 2012). Drug-related crimes have increased by 26.1 % over the past year in South Africa (South African Police Services, 2014), and methamphetamine, commonly known as 'tik', abuse is often linked to drug-related crimes. Furthermore, methamphetamine is the most common primary substance of abuse in the Western Cape (SACENDU, 2014).

Therefore, this research project aims to answer the following research question:
Can a method be developed in South Africa, which allows for the detection and quantification of methamphetamine and amphetamine in hair samples, using High Performance Liquid Chromatography – Tandem Mass Spectrometry?

2. Literature Review

The process of hair analysis involves at least five steps, as outlined by the Society of Hair Testing (SoHT) (Cooper, Kronstrand &Kintz, 2012):

1. washing
2. preparation of hair (drying, cutting into segments, pulverizing)
3. incubation
4. extraction
5. analysis

The SoHT has produced a set of guidelines in order to aid laboratories in hair testing and in an attempt to standardise various aspects of the hair analysis process. Even with these guidelines several procedures have been described over the past few years resulting in a wide range of published cut-off values for drugs and metabolites in hair, depending on the sensitivity of the methods used. The recommended cut-off concentration for amphetamines in hair, according to the SoHT, is 0.2 ng/mg of hair (Cooper, Kronstrand &Kintz, 2012).

Hair samples are washed prior to analysis in order to remove any hair care products, sweat or surface materials as well as to remove potential external contamination of drugs from the environment (Cooper, Kronstrand &Kintz, 2012). Washing procedures can vary in the solvents used, for example: proponal and phosphate buffer (Hegstad et al., 2008; Kronstrand et al., 2004); ethanol (Cheze et al., 2007) or sodium dodecyl sulphate (Miyaguchi et al., 2007), and the number of wash steps incorporated.

Following the washing and preparation steps hair undergoes an incubation step to release drugs from within the hair matrix (Cooper, Kronstrand &Kintz, 2012). Incubation can be carried out under varying conditions using different solvents such as methanol, hydrochloric acid, sodium hydroxide or a buffer such as phosphate buffer (Kintz, 2004). There are also varying methods for the drug extraction step which follows incubation, for example liquid-liquid extractions or solid-

phase extractions can be carried out (Kintz, 2004). Miyaguchi et al. (2007) developed an extraction method for the toxicological analysis of methamphetamine in hair using an automatic pulverizer. The pulverizing disintegrated the hair components and simultaneously allowed for the extraction of any drugs present in the hair. Only 2 mg of hair was required and after washing the method took less than 30 minutes to produce a chromatogram. Following extraction different analytical techniques are used for analysis of the drug extracts. These techniques range from immunoassays to methods involving gas or liquid chromatography coupled to mass spectrometry (Kintz, 2004).

Based on the increasing volume of literature being published on hair analysis for drugs it is evident that this is a valuable tool that has the potential to greatly benefit the field of forensic toxicology. However, to date, no research has been done in South Africa regarding the analysis of hair for drugs. Additionally, hair samples are currently not being collected during autopsies for the purpose of toxicological analysis. This project, therefore, has the potential to play a significant role in laying foundations for future hair analysis for drugs in South Africa.

3. Aims and Objectives

The aim of this project is to develop a method to screen for and quantify common drugs of abuse, namely amphetamine and methamphetamine, present in hair samples using High Performance Liquid Chromatography - Tandem Mass Spectrometry (HPLC-MS/MS).

This will be achieved through the following objectives:

1. set up a protocol for the collection of hair samples during suspected drug-related autopsies at the Salt River Mortuary, Cape Town
2. develop a method for the screening of drugs in hair samples using HPLC-MS/MS
3. develop a method to quantify amounts of amphetamine and methamphetamine detected in hair samples using HPLC-MS/MS
4. verify the screening and quantification methods developed by analysing authentic hair samples collected from the mortuary and comparing the amount of drugs detected in the hair samples to the amount of drugs detected in the respective blood samples also collected during autopsy.

4. Materials and Methods

4.1 Materials

4.1.1 Hair samples

Authentic hair specimens will be collected from the Salt River Mortuary during routine autopsies as part of service delivery. Hair samples (approximately a pencil thickness) will be cut from the posterior vertex region of the head as close to the scalp as possible. The hair will be placed into a collection envelope, sealed and labelled with a sticker recording the date, case number, pathologist, site of hair removal, colour of the hair and whether the hair has been chemically treated or not. The sealed envelope will be placed into a sealable plastic bag. The hair samples will be transported, along with other toxicology samples also collected, to the Toxicology Laboratory in the Pharmacology Division at the University of Cape Town. All hair samples will be stored at room temperature.

Hair specimens will also be collected from volunteers, from a drug centre, who have, within the last three months, taken drugs of abuse, specifically amphetamine or methamphetamine. These drug positive hair samples will assist in establishing a general method for the extraction of drugs in hair. Additionally, drug-free hair specimens, to be used for calibration, will be collected from volunteers who have no history of drug abuse and have not been on any chronic medication in the last year.

4.1.2 Instrumentation

Hair specimens will be pulverized using an Omni Bead Ruptor 24. A Shimadzu Prominence High Performance Liquid Chromatography System coupled to an API 3200 Q trap Mass Spectrometer, already being used for routine analysis in the Toxicology Laboratory, will be used for the screening and quantification of drugs in hair.

4.2 Preparation of hair extracts

4.2.1 Washing

Two different washing procedures will be tested on hair samples in order to develop a method that is the most effective in decontaminating the hair. In the first procedure hair samples will be washed once with ethanol and twice with dichloromethane for five minutes each. In the second

procedure hair will be washed once in an aqueous solution of Sodium Dodecyl Sulphate (SDS), twice with water and once with acetone for three minutes each. Samples will be dried at room temperature after washing. The last washing solution for each of the procedures will be concentrated and analysed by LC-MS/MS to check for the absence of drugs.

4.2.2 Drug extraction

Approximately 20 mg of hair, acetonitrile, 1M trifluoracetic acid (TFA), and an aqueous solution of the deutrated internal standards, as proposed by Miyaguchi et al. (2007), will be added to an Omni Bead Ruptor Lysis tube pre-filled with metal balls. The tubes will be capped and vigorously and uniformly shaken by the Omni Bead Ruptor 24 resulting in homogenization of the hair samples as well as drug extraction. After a brief centrifugation and filtration of the supernatant, the extract will be ready to be injected into the HPLC-MS/MS system.

4.3 Screening of authentic hair

A HPLC-MS/MS method will be developed and used for qualitative analysis of the extracts from the authentic hair samples.

4.4 Calibration

Calibration curves will be generated by spiking drug free hair with varying concentrations of reference standard.

4.5 Quantification

Hair samples that are positive for amphetamine or methamphetamine will be quantified using the calibration curves established in order to determine the amount of drug present in the sample of hair.

4.6 Data Analysis

Chromatograms will be analysed using Analyst 1.6.2.

The lower limit of quantification (LLOQ) will be determined for both amphetamine and methamphetamine in hair. The drug concentration will also be determined for each of the relevant authentic hair samples analysed. The amount of drug detected will be compared to the amount of drug detected in the respective blood samples collected from the same case. All data will be stored anonymously on a database with restricted access.

(The drugs amphetamine and methamphetamine will be provisionally looked at, however, if after initial screening of the authentic hair samples there is insufficient data then other common drugs of abuse will have to be considered.)

5. Ethics

For this study hair samples will need to be obtained from three different sources:

1. healthy volunteers, not on any chronic medication, and with no previous history of drug abuse,
2. volunteers who have, within the last three months, taken drugs of abuse, specifically amphetamine or methamphetamine, and
3. from suspected drug-related autopsies.

There are no ethical concerns surrounding the collection of hair as it will not result in any physical harm or create any health risks for volunteers. The scissors used to cut samples of hair from individuals will be sterile. Healthy volunteers, over the age of 18, will be recruited by approaching a hairdresser and asking suitable clients having their hair cut to donate a sample of their hair. Informed consent will be obtained from all volunteers.

Permission has been granted for volunteers over the age of 18, who are currently taking or have within the last three months taken drugs of abuse to be recruited from The Drug Counselling Centre in Observatory, Cape Town. The clinical psychologist at the centre will introduce the project to all suitable individuals and those who are interested in participating in the research will be put in contact with the researcher. Informed consent will be obtained from all of the

individuals willing to donate a sample of their hair. Hair samples will then be taken by the researcher, in a private setting within the Department of Pharmacology. All of the hair samples collected will be screened and only those samples that test positive for amphetamines will be quantified.

Hair from suspected drug-related autopsies will be collected from the Salt River Mortuary, Cape Town. Permission has been granted by Professor LJ Martin (Head of Clinical Department) for hair samples to be collected during autopsies along with other biological samples that are routinely collected as part of the Forensic Pathology Services service delivery. As with the hair samples collected from volunteers taking drugs of abuse, only those samples that test positive for amphetamines will be quantified.

No individual will be identified at any time as no names will be used in any of the results and all hair samples will be stored anonymously within a registered repository. Samples of hair will be stored indefinitely in the Division of Pharmacology at the University of Cape Town, however, no research unrelated to this project will be done without further ethics approval. The data generated from the analysis of hair samples will be stored anonymously in a database with restricted access requiring a password. No DNA analysis will be done on the samples of hair collected. All research will be conducted in a toxicology laboratory in the Division.

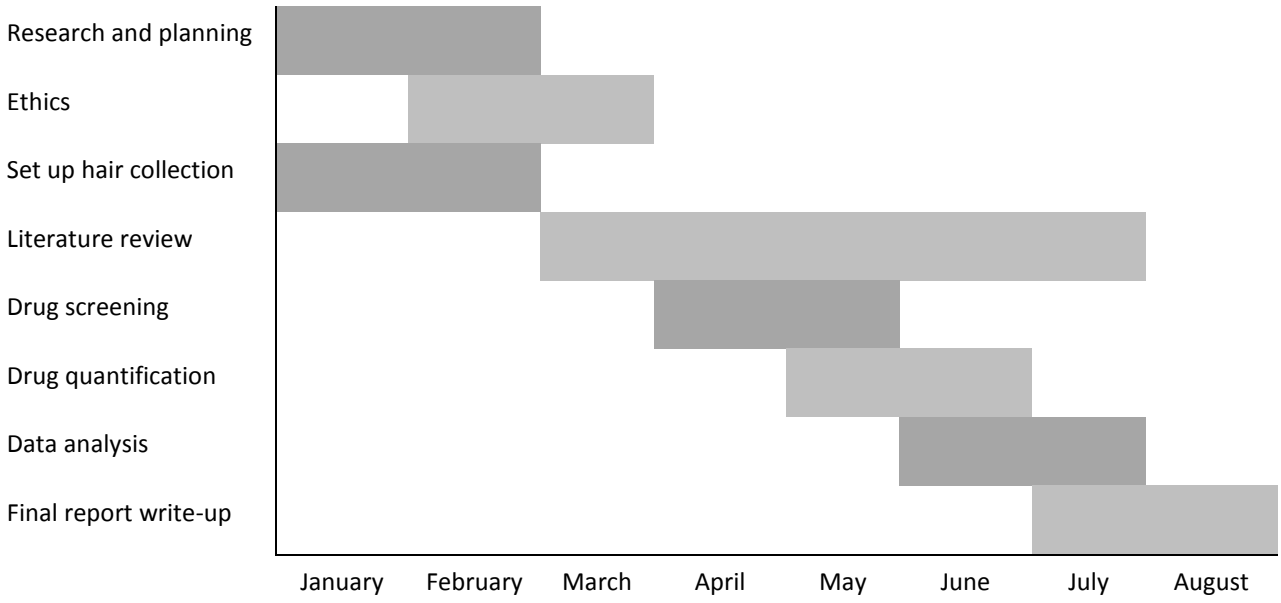
6. Sample numbers

There is no specific number of samples required from each of the three sources, as no statistical analysis will be done in this study. The hair collected from healthy individuals will be used as blank hair that will be spiked for calibration purposes. The hair samples from volunteers taking drugs will be used in assay development to confirm that the method developed is able to detect and quantify certain drugs. The samples of hair collected from autopsies will be used to verify the method developed and there is no exact number of samples needed in order to do this.

7. Budget

Description	Cost
Hair collection kit (~ 150 samples)	355
Compensation for volunteers	1 000
Running of instrumentation	5 000
Equipment (HPLC column)	7 000
Reagents (e.g. HPLC solvents)	2 000
Other chemicals (e.g. standards)	5 000
TOTAL	**20 355**

8. Work plan



Technical assistance will be provided by Alicia Evans (Division of Pharmacology).

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Addendum I: Adjustments to research proposal

As the study progressed, minor changes were made to the methodology described in the research proposal. These changes were mainly due to time constraints and are outlined below:

Analytes of interest

The reference standard purchased for the study contained a mix of six amines, including amphetamine and methamphetamine. MDMA was also included in the mix and it was decided, as MDMA is also a common drug of abuse in South Africa, to include it in the method.

Development of quantification method for blood samples

Before the start of the study there were no methods in place for the quantification of amphetamines in blood. The results from the analysis of forensic blood samples were required for comparison purposes. It was therefore necessary to develop sample preparation and HPLC-MS/MS methods for the detection and quantification of amphetamines in blood.

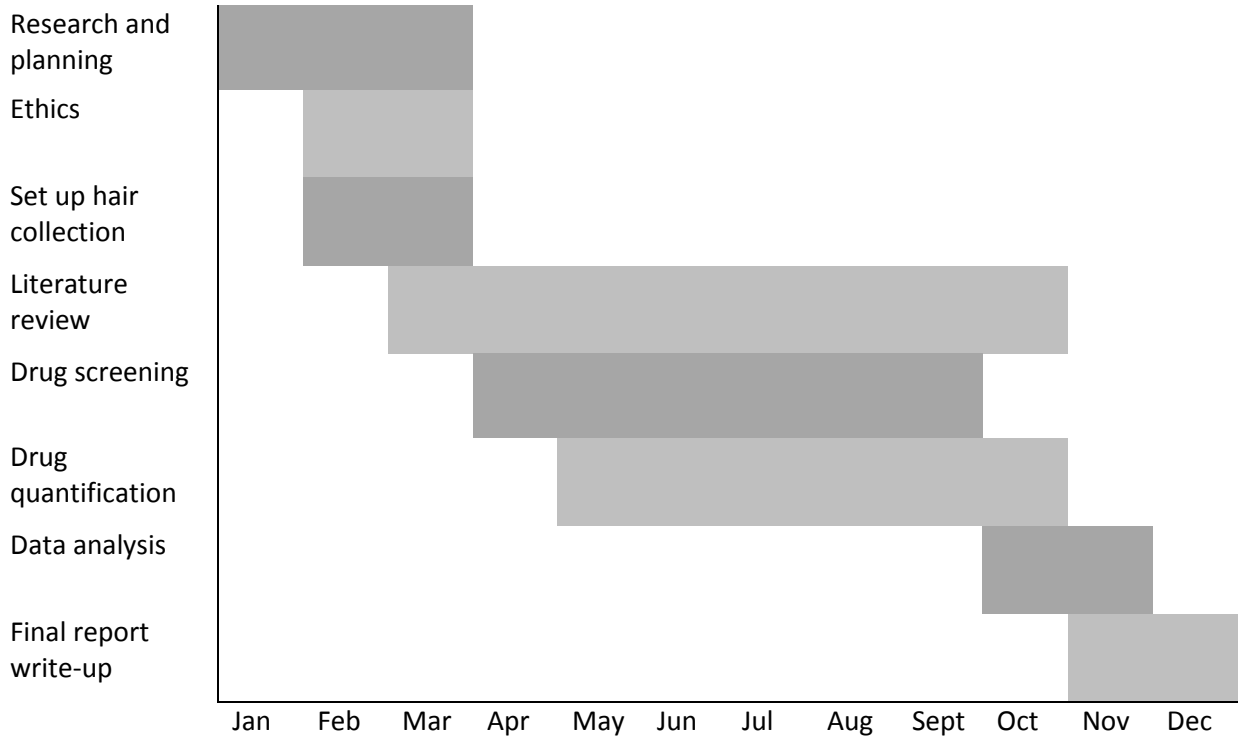
Determination of LLOQ

Time constraints did not allow for the determination of LLOQ's for the analytes of interest in blood and hair. As a result LLOQ's were set according to the sensitivity of the instrument.

Screening of authentic hair samples

According to the proposal, all authentic hair samples (collected from drug users and suspected drug-related autopsies) were meant to be screened to detect for the presence of amphetamine, after which any amphetamine positive samples would be quantified. However, due to time constraints this was not possible. As a result, only hair samples collected from drug users who admitted to amphetamine use were analysed. Toxicological samples, such as blood and urine, collected from autopsy cases are routinely screened for the presence of drugs by the laboratory. From the screening results, only amphetamine positive cases for which both blood and hair samples were available were analysed and quantified as a part of this study.

Revised work plan



Chapter 2: Structured Literature Review

Overview of hair analysis for drugs and the limitations associated with the interpretation of results

1. Introduction

Traditionally, drug analysis has involved the use of blood and urine as biological matrices, however, since the first report on hair analysis in 1979 (Baumgartner et al., 1979), there has been an increased interest in research surrounding hair as both an alternative and complementary approach for drug analysis (Miller, Wylie & Oliver, 2008). Hair was first analysed for drugs in 1979 by Baumgartner et al. to detect morphine in the hair of heroin abusers using a radioimmunoassay method (Baumgartner et al., 1979). More recent methods developed to detect drugs in hair involve analytical techniques such as chromatography and mass spectrometry (Pragst & Balikova, 2006). Hair differs from other biological matrices, such as blood and urine, in that it allows for a long detection window (months to years) for drugs, long after the drug has been eliminated from the rest of the body (Wada, 2006). Therefore, whilst urine and blood analysis provide short-term information of an individuals drug use, through hair analysis a long-term history can be determined (Kintz, 2004).

In addition to a long detection window, there are many other advantages to hair analysis, namely: the process of sample collection is simple and non-invasive, samples are easily stored and transported, hair does not decompose like other bodily fluids and hair samples cannot easily be tampered with (Cooper, Kronstrand & Kintz, 2012). Moreover, segmental analysis of hair helps to provide a historical profile of an individual's drug history (Balíková, 2005). As a result, hair analysis for drugs has many different practical applications. Examples of these include criminal investigations involving drug-related deaths and drug-facilitated crimes, as well as for monitoring the misuse of drugs in the workplace, drug testing or drug treatment programs (Cooper, Kronstrand & Kintz, 2012).

Despite the many advantages and applications of hair analysis, this approach is complicated, and developing methods for the detection and quantification of drugs in hair can be challenging. Hair analysis generally involves various steps concerning the collection, preparation and analysis of hair samples, however, there is no consensus on the exact methodology to be followed (Cooper, Kronstrand & Kintz, 2012). Additionally, there are limitations to this type of analysis that not only make the interpretation of hair analysis results difficult but also question the validity of these results.

In order to assist with the challenges and limitations of hair analysis, the Society of Hair Testing (SoHT) has established recommended guidelines for hair analysis as well as cut-off

concentrations for drugs in hair to identify drug use (Cooper, Kronstrand & Kintz, 2012). Consequently, numerous articles have been published over the years reporting various different methods for the analysis of hair for several different substances. Moreover, it is evident from research that there is an increase in the number of laboratories currently performing hair analysis on samples of hair that are routinely collected for toxicological purposes (Kintz, 2004). Despite these advances, hair analysis for drugs is currently not performed in South Africa. The spectrum of biological samples collected by pathologists during routine autopsies of suspected drug-related deaths in South Africa may include blood, urine, stomach contents, bile or vitreous fluid. However, at present, no hair samples are being collected for analysis.

Drugs are abused on a daily basis throughout South Africa, and according to recent crime statistics drug-related crimes have increased by 26,1 % over the past year (South African Police Services, 2014). In the Western Cape, methamphetamine, commonly known as 'tik' in South Africa, is the most common primary substance of abuse (SACENDU, 2014), and over the last decade there has been a dramatic increase in the use of 'tik' (Watt et al., 2014). A qualitative study was conducted on members of a Cape Town community to determine the impact of 'tik' abuse on the community. The members of the community who were interviewed as part of the study linked 'tik' use to increased rates of crime, violence and corruption (Watt et al., 2014). The monitoring as well as detection and analysis of drugs, therefore, plays a vital role in providing more information surrounding cases of drug-related crime and death in South Africa. Moreover, there is great potential for hair analysis to make a significant contribution to the advancement of forensic toxicology in South Africa.

Therefore, the aim of this review is to provide insight into some of the various methodologies that have been developed for the analysis of hair for drugs. This review will provide a brief overview of the anatomy and physiology of hair including the mechanisms by which drugs are incorporated into hair. The interpretation and limitations of hair analysis results will also be discussed. Literature was searched for on Google Scholar using the keywords: hair, amphetamine, liquid chromatography mass spectrometry, post-mortem toxicology. Publications were chosen between the years 1993 – 2014.

2. Amphetamines

Methamphetamine is metabolized to amphetamine *in vivo*, and these two compounds comprise the majority of abuse seen within this class of drugs (Cody, 1993). Amphetamine and amphetamine type stimulants are weak basic compounds with low molecular weights. These characteristics allow these compounds to diffuse easily across cell membranes and lipid bilayers (de la Torre et al., 2004). During drug analysis, both amphetamine and methamphetamine concentrations are usually measured and reported (Curtis, 2008). Concentrations of amphetamine and methamphetamine measured in blood greater than 0,2 and 0,15 mg/L respectively are considered toxic, and possibly fatal (Schulz et al., 2012). The chemical structures of amphetamine and methamphetamine are shown (Figure 1).

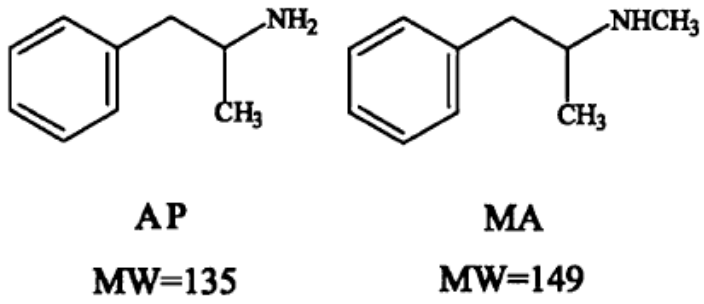


Figure 1: Chemical structures and molecular weights (MW) of amphetamine (AP) and methamphetamine (MA). (Reproduced from Tabernero et al., 2009)

Amphetamines are powerful stimulants of the central nervous system and cause increased alertness, insomnia, energy and self-confidence as well as a decrease in appetite (de la Torre et al., 2004). Compounds within this class of drugs can be prescribed and used as legitimate medication or taken as drugs of abuse (Musshoff, 2000), however, due to the high potential for abuse the medical uses have been limited (Concheiro et al., 2007). Presently, there are as many as 14 precursor compounds of amphetamine and methamphetamine (Musshoff, 2000; Cody, 1993). This complicates the interpretation of amphetamine results, as a positive result can occur either from the use of legitimate medication or as a result of illicit drug use. A positive test result, therefore, is not always adequate to explain the source of the amphetamine exposure (Curtis, 2008).

3. Anatomy and Physiology of Hair

Hair is a complex structure and in order to accurately interpret and understand the results of hair analysis tests it is important to have some understanding of the anatomy and physiology of hair (Harkey, 1993). Most of the human body is covered with hair, however, depending where on the body the hair is located, the composition, thickness and growth rate of the hair may vary (Curtis, 2008).

Hair is typically composed of cylindrical shafts made up of tightly compacted cells that grow from follicles (Figure 2). Hair follicles are located in the epidermis and are surrounded by dense capillary networks. There are three types of glands that are closely associated with hair follicles, namely the sebaceous, apocrine, and sweat glands, depending on the site of the hair. The secretions of these glands cover the hair shaft and therefore make it possible for drugs to be transferred into hair through these secretions (Harkey, 1993).

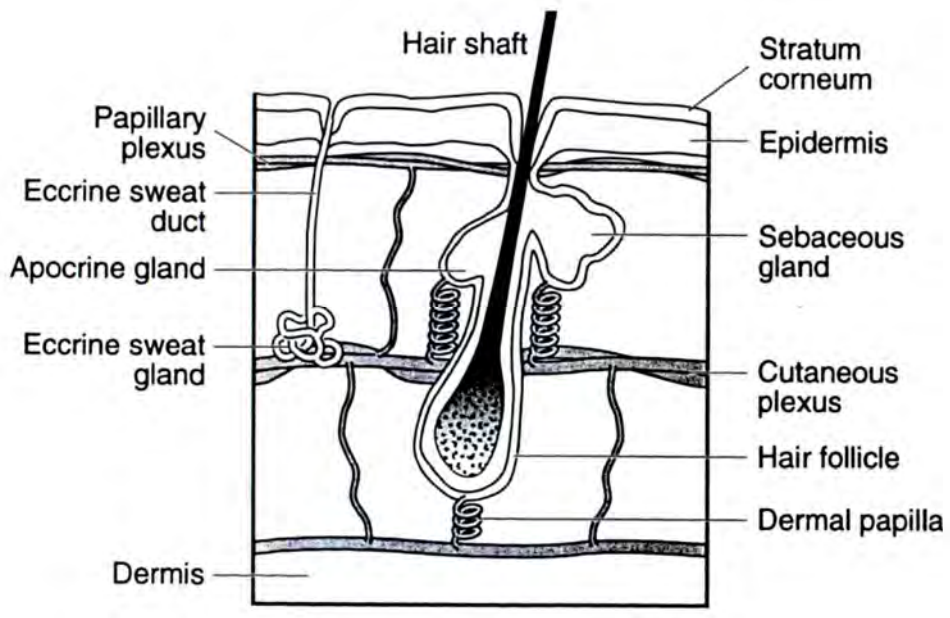


Figure 2: Simplified diagram of a hair follicle, the associated apocrine, sebaceous and sweat glands, and the dense capillary networks located in the surrounding tissue. (Reproduced from Harkey, 1993)

An individual hair shaft can range from 15 to 120 µm in diameter. As can be seen in Figure 3 below, each hair shaft is comprised of an outer cuticle, an inner cortex and a central medulla. The cuticle, which functions to protect the inner fibers, is often damaged or destroyed by

chemicals, heat or light. The color of human hair is determined primarily by the amount and distribution of a pigment, melanin, which is found in cortical cells (Harkey, 1993). Melanin provides binding sites for certain drugs. This introduces the potential for bias in hair testing as it is possible that drugs may be detected in higher concentrations in individuals with darker hair (Curtis, 2008).

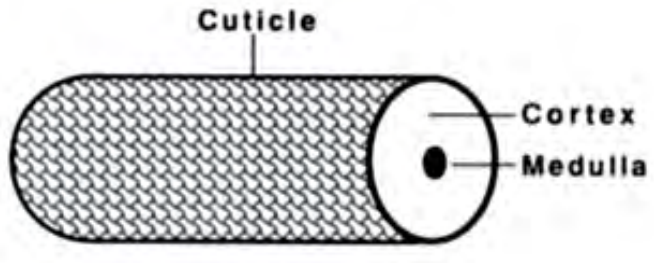


Figure 3: Cross-section of a hair shaft illustrating the cuticle, cortex and medulla. (Reproduced from Harkey, 1993)

Hair does not grow continuously, but rather in cycles of three phases. The first phase is the anagen phase, which lasts between 4 – 6 years, and is a phase of active growth (Harkey, 1993). During this phase nutrients are delivered to the follicle by the capillary network. Additionally, any extraneous substances found in the blood, such as trace metals or drugs, for example amphetamine, are also incorporated into the hair shaft. As the hair shaft grows these substances are moved along the shaft (Bost, 1993). The second and third phases of the hair growth cycle are the catagen (transition) and telogen (resting) phases, which last a few weeks and 4 – 6 months respectively (Harkey, 1993). As not all hair grows at one specific time, it is difficult to determine the exact amount that hair grows in a given time period. It is usually assumed, therefore, that the average rate of growth for human head hair is one centimeter per month. The most important factors that determine the rate of hair growth are the type and the location of the hair. For example, scalp hair grows more quickly than pubic or axillary hair whilst beard hair grows the slowest. Other factors that further affect growth rate are race, sex and age (Harkey, 1993).

Scalp, beard, axillary and pubic hair are all considered suitable samples for hair analysis. Although, biological differences between these types of hair, such as growth rate, need to be considered when interpreting and comparing hair analysis results. Scalp hair samples are the easiest to collect and are considered to be the best sample for drug analysis. Ideally hair samples

are collected from the posterior vertex region of the scalp as this is where the growth rate is the most constant (Harkey, 1993). In the absence of head hair, however, or if the hair is too short, it becomes necessary to use samples of hair from other parts of the body.

In a study conducted by Han et al. (2005), to compare the concentration of methamphetamine between head, axillary and pubic hair, samples of hair were collected from these each of these regions from suspected methamphetamine users. A comparison of the hair analysis results showed that the concentrations of amphetamine and methamphetamine were higher in axillary and pubic hair than in head hair (Han et al., 2005). A potential advantage of using hair samples from these regions is that there is less external contamination of the hair. Pubic hair, however, can easily be contaminated by urine (Curtis, 2008), which may account for the increased drug concentration found in this region.

4. Mechanisms of drug incorporation into hair

Whilst there is still uncertainty on the exact mechanisms by which drugs are incorporated into hair, two models have generally been accepted into practice. The simpler model is a passive transfer model whereby drugs are incorporated into hair through passive diffusion from the bloodstream. The drugs are incorporated into the growing cells of the hair follicle and become tightly bound in the interior of the hair shaft (Henderson, 1993). However, this model is oversimplified and a multi-compartment model, proposed by Henderson (1993), is generally accepted to be more accurate.

Henderson (1993) suggests, in this multi-compartment model, that drugs may be incorporated into hair through three different mechanisms, namely from the bloodstream during hair formation; through the secretions of the sweat, sebaceous and apocrine glands during hair formation; and from the external environment after the hair has formed. Figure 4 illustrates the various mechanisms through which drugs can be incorporated into hair as well as two mechanisms through which drugs can be eliminated from hair.

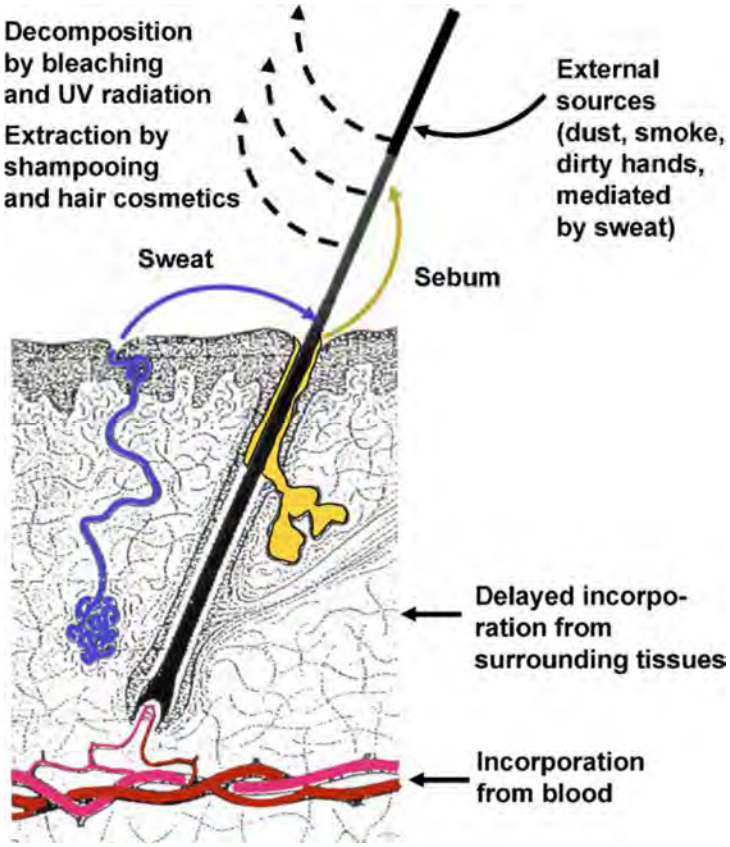


Figure 4: Mechanisms of drug incorporation (solid arrows) and elimination (dotted arrows) in hair. Besides incorporation from blood, drugs can also be incorporated from deep skin compartments during the formation of the hair shaft. Drugs can also be deposited by diffusion from sweat or sebum secretions into the hair follicle, and from the external environment. (Reproduced from Pragst and Balikova, 2006)

The physiochemical properties of drugs are also an important factor to consider as they are known to affect the rate of drug incorporation into hair. Research conducted by Nakahara et al. (1995) described the effect of three physiochemical properties; lipophilicity, melanin affinity and membrane permeability, on drug incorporation into hair. Lipophilicity and basicity were found to be the main factors favoring drug incorporation, and generally basic drugs, such as amphetamine, had higher incorporation rates than neutral or acidic drugs. Methamphetamine, the parent compound, is less polar than the metabolite, amphetamine, and the study also showed that as a result methamphetamine has a higher incorporation rate and is the predominate analyte found in hair (Nakahara, Takahashi & Kikura, 1995).

5. Analytical methods used for hair analysis

Several different methodologies have been described for hair analysis for drugs over the past few years, making the comparison of results difficult. In an attempt to standardise various aspects of the hair analysis process, the SoHT, has produced a set of guidelines in order to assist laboratories in hair testing. Although, even with these guidelines a wide range of cut-off values for drugs and metabolites in hair have been published, depending on the sensitivity of the methods used (Cooper, Kronstrand & Kintz, 2012). The steps that are generally followed during hair analysis are outlined in figure 5 and will be discussed further in this section.

5.1 Collection and storage of hair samples

As mentioned previously, samples of hair collected from an individual may include beard, arm, leg or pubic hair, however, head hair is generally preferred. Samples of hair, usually equivalent to a pencil thickness, are cut from the posterior vertex as close to the scalp as possible (Cooper, Kronstrand & Kintz, 2012). The guidelines suggested by the SoHT recommend that head hair samples should be aligned with the root end of the hair clearly marked. The most common method for storing hair samples is to wrap the sample of hair collected in aluminum foil and to then place it in a labeled paper envelope. Finally, dry hair samples should be stored in a dry, dark environment at room temperature (Cooper, Kronstrand & Kintz, 2012).

An important aspect of hair analysis is to record the necessary information, especially if the collected hair is to be used for forensic purposes. This information includes; the identification of the person from whom the sample was collected from as well as characteristics of the hair sample such as the mass, length, colour, site of collection and any cosmetic treatments, such as bleaching (Cooper, Kronstrand & Kintz, 2012).

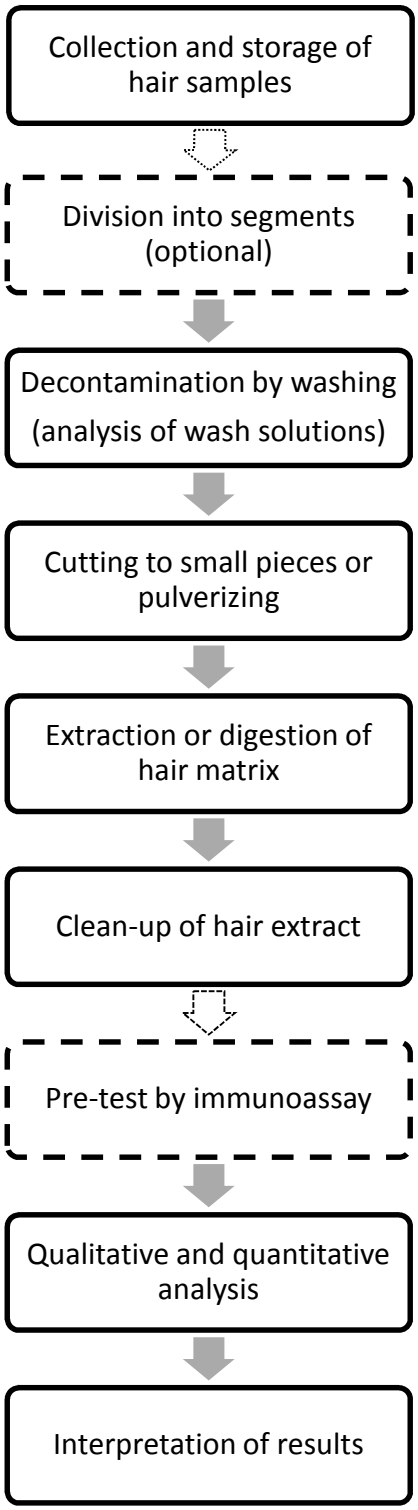


Figure 5: Steps in the forensic analysis of hair for drugs. Dotted lines represent optional steps. (Adapted from Pragst and Balikova, 2006)

5.2 Division into segments

Segmental analysis involves cutting samples of hair into measured segments. The length of segments varies and different strategies for segmentation apply depending on the individual case. According to the SoHT, segments between 10 and 30 mm are acceptable, although, if necessary shorter segments of hair may be used (Cooper, Kronstrand & Kintz, 2012). Since the drugs become incorporated into the hair shaft and are moved along the shaft as the hair grows, segmental hair analysis has the potential to provide a detailed history of drug abuse for an individual. Furthermore, this type of analysis may distinguish a single drug exposure from long-term exposure (Pragst & Balikova, 2006). Using small segments of hair is particularly useful in drug facilitated crimes that involve the single administration of a specific drug, often seen in cases of drug facilitated sexual assault. In these cases, it is possible to determine a positive hair result corresponding to a narrower timeline (Cooper, Kronstrand & Kintz, 2012).

5.3 Decontamination

Hair samples are washed before analysis in order to remove any hair care products, sweat or sebum that may interfere with the analysis as well as to remove any contaminants from the external environment. Decontamination of hair samples is essential as passive exposure to drugs in the environment may result in a false positive test result (Musshoff, 2007). Wash steps are therefore included in the hair analysis process in order to remove any external contamination from the surface of the hair, although, it is possible that over washing might result in drugs incorporated in hair to be removed (Nakahara, 1999).

There is no consensus as to how hair samples should be washed and there are several different approaches that are discussed throughout the literature. Organic solvents, such as dichloromethane, only remove any surface contamination from the hair. Aqueous solutions, or methanol, on the other hand cause the hair to swell and result in drugs from within the hair matrix to be extracted. The SoHT recommends that washing procedures include steps with both organic solvent and aqueous solutions (Cooper, Kronstrand & Kintz, 2012).

The most common wash solvents used by laboratories include proponal and phosphate buffer (Hegstad et al., 2008; Kronstrand et al., 2004), ethanol (Chèze et al., 2007); sodium dodecyl sulphate (Miyaguchi et al., 2007) and dicholormethane (Koster et al., 2014). The duration of the washes, as well as the number of wash steps is determined by individual laboratories. It is

suggested by the SoHT that laboratories investigate to what extent their wash procedure removes any surface contamination (Cooper, Kronstrand & Kintz, 2012). Most methods for washing developed by laboratories include the analysis of wash solvents in order to check for external contamination (Koster et al., 2014; Montesano, Johansen & Nielsen, 2014). However, as there is not yet a standardised method for the effective decontamination of hair, the possibility of external contamination cannot be ignored when interpreting hair analysis results.

5.4 Drug extraction

In order for drugs present in hair to be detected, identified and quantified, these substances must first be dissolved into a solution (Curtis, 2008). Depending on the extraction method used, the resulting extract can either be analysed directly, or will require further clean-up steps using either liquid-liquid extraction or solid-phase extraction. As with the washing step, there are several ways to extract drugs from hair. The efficiency of the method used, however, can be significantly compromised by using unsuitable extraction procedures not targeted to specific drugs (Cooper, Kronstrand & Kintz, 2012). For most methods the amount of hair used ranges from 10 to 20 mg.

Methanol is often used as an extraction solvent, and methanolic extraction in an ultrasonic bath is compatible with almost all drugs. The ultrasonication causes the hair structure to degrade, and the methanol penetrates the hair matrix causing the hair to swell and drugs to be extracted (Musshoff, 2007). The extraction time may vary, however, a mistake that is often made is the use of an extraction time that is too short. Drug recovery is often incomplete when methanolic ultrasonication is used, and recoveries are often lower than recoveries obtained when other procedures are used (Musshoff, 2007). Another disadvantage of this approach is that there is a relatively high level of impurities in the extract and a clean-up step is generally required (Pragst & Balikova, 2006).

Other procedures involve the use of an acid, such as hydrochloric acid (HCl), or an alkali, such as sodium hydroxide (NaOH) to digest the hair matrix, thereby releasing any incorporated drugs into solution (Curtis, 2008). Acidic methanol has been reported to be a good extraction solvent for the extraction of amphetamines from hair, and methods commonly used involve extraction with methanol/5 M HCl (20:1), with a sonication step (Nakahara, 1995). Another approach to drug extraction is enzymatic treatment which involves the use of enzymes for the destruction of the hair structure. Whilst these methods are advantageous as they solubilise the hair sample

without causing any unstable compounds to degrade, they are relatively expensive (Nakahara, 1999).

It has been previously reported that alkaline methods, such as digestion with aqueous NaOH, provides high yields for drugs, such as amphetamine, that are stable in an alkaline environment (Musshoff, 2007; Nakahara, 1999). Kintz and Cirimele (1997) compared various approaches for the extraction of amphetamine and related compounds from a sample of head hair collected from a known drug abuser who overdosed on methylenedioxymethamphetamine (MDMA). Extractions were performed using either alkaline, enzymatic or acid hydrolysis or methanol sonication. The results showed that the best recoveries were obtained after alkaline hydrolysis (Kintz & Cirimele, 1997). It has also been reported, however, that basic drugs, such as amphetamine, are also well extracted by a neutral or slightly acidic aqueous buffer, such as phosphate buffer, or aqueous HCl (Musshoff, 2007).

The various approaches mentioned above can be time consuming, and more recently researchers are incorporating a pulverization step into the extraction procedure ((Koster et al., 2014; Montesano, Johansen & Nielsen, 2014). After adding an extraction solvent to samples of hair, the hair is pulverized. Pulverization increases the surface interaction of the hair and extraction solvent, resulting in the simultaneous disintegration of the hair and extraction of drugs. A pulverization method, is therefore, not only less time consuming, but also creates a more homogenous sample and does not result in any chemical instability in the drugs being extracted (Koster et al., 2014).

Research conducted by Miyaguchi et al. (2007) describes one of the first extraction methods to be developed using pulverization. This method was used for toxicological analysis of head hair that was collected from methamphetamine users. Two milligram samples of hair were pulverized for five minutes together with an aqueous solvent containing 10 % acetonitrile and 100 mM trifluoroacetic acid. The method took less than 30 minutes to obtain a HPLC-MS/MS result from a washed hair sample (Miyaguchi et al., 2007). By pulverizing the hair, the researchers attempted to destroy the physical structure of hair in order to facilitate the extraction. Figure 6 shows images of pulverized residues from a sample of blank hair after being pulverized for various periods of time. After one minute of pulverization the cuticle layer was greatly damaged, and pulverization for more than three minutes caused the cortical fibers to disintegrate. As previously discussed, melanin granules, which are the main binding site for basic drugs, are contained within the cortical cells (Harkey, 1993). The disintegration of these fibers,

therefore, strongly facilitates the extraction of basic drugs. The amount of drug extracted after each of the different pulverization times was determined and the results showed that after three minutes of pulverization the extraction was complete (Miyaguchi et al., 2007).

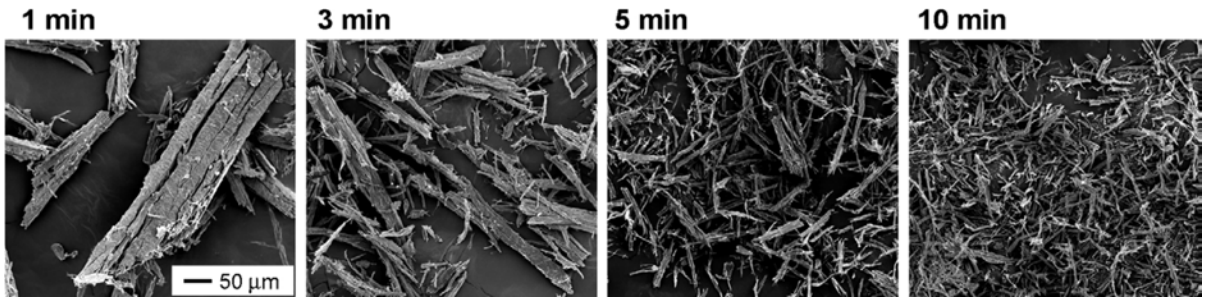


Figure 6: Scanning Electron Microscope images of drug free hair after being pulverized for one, three, five and ten minutes. (Reproduced from Miyaguchi et al., 2007)

5.5 Pre-test by Immunoassay

Immunoassays are generally used during drug testing as an initial or preliminary screen. The screening of samples for a range of drugs functions to divide samples into negative and potentially positive specimens (Pragst & Balikova, 2006). The first hair analyses for drugs were performed by radioimmunoassay (Baumgartner et al., 1979), however, immunoassays are not considered a definitive method due to concerns of false positives as a result of the lack of specificity characteristic of this type of technique. Positive results must be confirmed by a separate technique, such as GC-MS (Curtis, 2008). Additionally immunoassay drug testing kits originally developed for urine are not sensitive enough to be used in hair analysis (Pragst & Balikova, 2006).

5.6 Chromatography and Mass Spectrometry

Chromatographic techniques coupled with mass spectrometry can also be used for drug screening purposes. Moreover, these techniques can be used to identify and quantify drugs. There are a number of different analytical approaches towards analysing hair for drugs. Various options exist when developing a method; such as the type of chromatography and mass spectrometry to be used, the column, mobile phases, gradient, flow rate, collision energy as well as several other chromatography and mass spectrometry conditions and parameters. This wide

range of variability between methods makes it difficult to compare results, and is one of the reasons why the SoHT has developed guidelines and cutoff concentrations for drug testing in hair (Cooper, Kronstrand & Kintz, 2012).

Gas Chromatography – Mass Spectrometry (GC-MS) is the analytical method that is used most often for hair analysis, and is more sensitive, specific and selective compared to other analytical methods (Nakahara, 1999). However, gas chromatography requires a specific chemical derivatization step, which can be complicated and create issues with the stability of some compounds (Pragst & Balikova, 2006). In order to avoid a derivatization step, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is being used more frequently in forensic toxicology for the screening and quantification of a wide range of compounds (Montesano, Johansen & Nielsen, 2014). To obtain high levels of selectivity, two fragments (a quantifier and qualifier) are monitored simultaneously. The quantifier (most intense fragment) is used to calculate concentrations and the qualifier (second fragment) is used for confirmation of the detected substance (Koster et al., 2014).

Several LC-MS/MS methods have been developed over the past decade that have been used to successfully analyse amphetamines, as well as numerous other compounds in hair (Fernández et al., 2014; Jakobsson & Kronstrand, 2014; Montesano, Johansen & Nielsen, 2014; Favretto et al., 2011; Nielsen et al., 2010; Tabernero et al., 2009; Hegstad et al., 2008; Chèze et al., 2007; Kronstrand et al., 2004). Kronstrand et al. (2004) developed a method for the simultaneous analysis of several drugs of abuse, including amphetamine, methamphetamine and MDMA, using ion spray LC-MS/MS. Head hair from autopsy cases was analysed in addition to blood and urine specimens that were collected. The results of the study showed high sensitivity and, according to the researchers, proved appropriate for screening purposes (Kronstrand et al., 2004).

A method using LC-MS/MS for the simultaneous analysis of six amphetamines in hair, blood and urine has also been developed (Chèze et al., 2007). This highly sensitive method was applied to a forensic case for the determination of MDMA after a single administration of ecstasy. Urine and blood samples were collected eight days and head hair two months after ingestion. No MDMA was detected in the blood, but both the urine and hair tested positive for MDMA. Segmental hair analysis was carried out on the hair sample and 22 pg/mg MDMA was detected; only in the segment of hair corresponding to the period of time when the ecstasy was ingested (Chèze et al., 2007).

More recently, ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) methods have been developed and validated for the quantification of drugs in hair (Fernández et al., 2014; Jakobsson & Kronstrand, 2014; Montesano, Johansen & Nielsen, 2014). The introduction of UHPLC has expanded the number of analytes that can be screened for in a single chromatographic run to more than 100. Montesano et al. (2014) recently developed and validated a UHPLC-MS/MS method for the targeted analysis of 96 drugs and drugs of abuse in hair. The limit of detection and quantification for amphetamine, methamphetamine and MDMA was determined to be as low as 0.002 and 0.005 ng/mg respectively (Montesano, Johansen & Nielsen, 2014).

5.7 Interpretation of results

"The most serious pitfalls of hair analysis are not in the practical performance but in the interpretation of results" (Pragst, 2004). Accurate interpretation of hair analysis results involves determining the answers to various questions surrounding the individual case as well as the analytical procedure. Some of these questions include:

- Did the individual use drugs, or is the positive result due to external contamination?
- Which drugs were used?
- Was it single, occasional, regular or excessive drug use?
- When were the drugs used?
- What is the limit of detection of the analytical procedure?
- How specific is the analysis?

A great deal of literature has been published surrounding the best approach to answering these questions (Musshoff, 2007; Pragst & Balikova, 2006; Kintz, 2000; Wennig, 2000; Nakahara, 1999; Kintz, 1995; Bost, 1993). One of the most important questions to consider when interpreting results is what the concentration of a certain drug or metabolite means. Research suggests that there is only a limited correlation between how often drugs are used or the amount of drug that is used and the concentration of drug found in hair between individuals (Musshoff, 2007). According to Kintz et al. (2000), this lack of correlation can be explained by a number of factors including variability in the rate of hair growth, the stability and retention of drugs in hair that has undergone chemical treatment, uncertainty of the amount of drug ingested by abusers and multiple mechanisms by which drugs can be incorporated into hair.

6. Quality Control

The establishment of suitable cut-off values and implementation of quality control measures are essential when it comes to accurately interpreting hair analysis results. Cut-off values allow for the correct identification of drugs or metabolites in hair (Wennig, 2000). The recommended cut-off value for amphetamines, according to the SoHT, is 0,2 ng/mg (Cooper, Kronstrand & Kintz, 2012). In comparison to blood and urine, quality control for quantitative analysis in hair is generally more difficult. This is mainly due to the solid nature and complex composition of hair as well as the insufficient amount of authentic reference material available (Pragst & Balikova, 2006).

Interlaboratory studies, or precision tests, are regularly performed by laboratories as a process by which quality control is monitored. The same homogenous sample of hair is sent to various laboratories for drug identification and quantification, and the results from each laboratory are then compared. In the past, these types of studies have reported inconsistent results, mainly for quantitative analysis (Kintz & Cirimele, 1997; Welch, Sniegoski & Allgood, 1993). It has become evident, from studies such as these that further work needs to be done in order to ensure quality in hair testing, which is one of the goals of the SoHT (Sachs, 1997). As hair provides more information than other biological samples used for drug analysis, hair analysis results, and the correct interpretation thereof, are of great importance. This is especially true when the results are to be used in court cases. Quality assurance and control in hair analysis therefore becomes a major concern in forensic toxicology cases (Lee et al., 2008).

There are three different approaches to the type of hair specimen that is used for quality control purposes, namely:

1. spiked hair, which involves the spiking of drugs onto blank hair (as done with liquid matrices);
2. fortified hair, which involves the soaking of blank hair with drug analytes for several days allowing for the drugs to be incorporated into the hair; and
3. authentic hair, collected from drug users, which contain the drug of interest (Turfus et al., 2013).

Spiked hair is most commonly used for quality control in hair analysis, however, the process of spiking hair does not model the process by which drugs are incorporated into hair. It is unknown

how much of the drug is actually incorporated into the hair as most of the drug will lie on the surface of the hair (Figure 7) (Sachs, 1997).

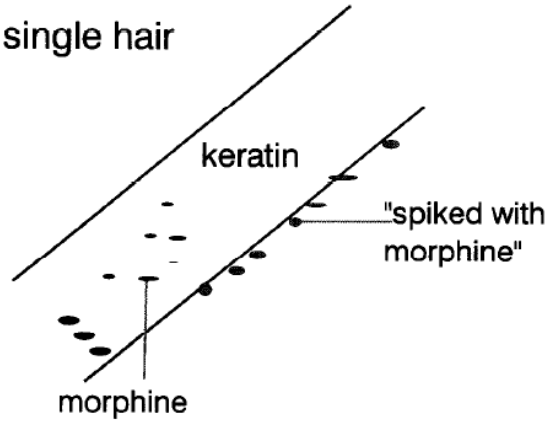


Figure 7: A simplified illustration of the difference between drugs that have been incorporated into the hair matrix, through ingestion, and drugs that lie on the surface of the hair, through spiking. (Reproduced from Sachs, 1997)

Fortified hair has been recommended as an alternative to spiking (Cooper, Kronstrand & Kintz, 2012), and this approach has been used to develop reference standards for the analysis of amphetamine and methamphetamine (Lee et al., 2008). As they have certified values of drug analytes, reference materials are useful for method development and validation as well as internal quality control. Welch et al. (2003) developed two standard reference materials, each for a different range of drugs, using dimethylsulfoxide. Dimethylsulfoxide has the ability to penetrate the hair matrix and is therefore useful for causing drugs to be incorporated into hair. For each reference standard, about 30 g of drug free hair, collected from a single individual, was soaked in the respective analytes, to which dimethylsulfoxide was added, for 16,5 days (Welch, 2003). The concentration of each analyte in the reference standards was determined by using both GC-MS and LC-MS. Even though there is no way to know exactly how much of the drug was absorbed by the hair, the GC-MS and LC-MS results compared well and mean differences for the analytes ranged from 4 to 16 %.

In the case of authentic hair samples, drugs have been ingested and therefore incorporated into the hair matrix, however, it is difficult to know the exact concentration of the drug that is in the hair sample (Turfus et al., 2013). Turfus et al. (2013) performed a comparison of quality controls prepared from spiked, fortified and authentic hair for the determination of ethyl glucuronide.

Figure 8 outlines the different methods used in the preparation of each of these types of quality controls.

The results of this comparative study showed that spiked quality controls resulted in better repeatability and inter-assay precision. Despite these results, the authors recommend that authentic or fortified hair samples be used for quality control as they are more representative of case samples (Turfus et al., 2013).

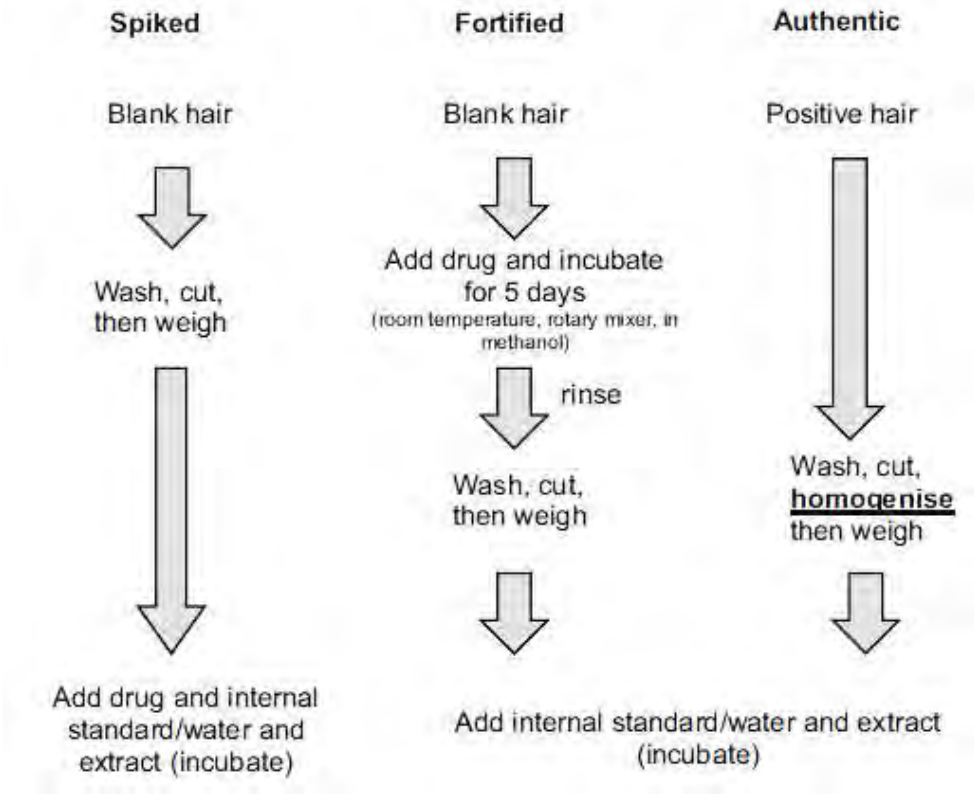


Figure 8: An outline illustrating the processes followed for the preparation of the three different types of quality controls that were used in this comparative study. (Reproduced from Turfus et al., 2013)

7. Limitations of hair analysis

As previously discussed, hair is one of the most variable biological matrices and the problems associated with accurately interpreting hair analysis results are made more challenging by various limitations that are associated with this method of drug analysis. A few of these limitations are discussed below:

7.1 External Contamination

Unlike blood or urine, hair is exposed to the external environment and is therefore exposed to contaminants, such as drugs, that might be present in the environment. External contamination is one of the main limitations as false positives may arise as a result of external contamination (Baumgartner & Hill, 1993). Baumgartner and Hill (1993) describe several different approaches to determine whether a positive test result is due to active drug use or as a result of passive contamination. These include: washing hair samples before analysis, analysis of the wash solution to compare the concentration of drug in the washes to the concentration in the extracted hair sample, establishing cut-off values and determining drug metabolite concentration ratios.

7.2 Colour and Racial Bias

As previously mentioned several drugs have the tendency to bind to melanin (Curtis, 2008). More specifically, this pigment plays an important role in the binding of amphetamines into the hair shaft. Studies have shown that the incorporation of amphetamine and methamphetamine into hair is affected by hair colour (Kronstrand et al., 2003; Kronstrand et al., 2001). In these studies the melanin content in hair was determined using spectrophotometry, and results showed an increase in the amount of methamphetamine detected with increased melanin content. In a recent study a method was developed for the segmental analysis of amphetamines in hair using UHPLC-MS/MS. When comparing the amount of amphetamine detected in samples of hair with hair colour, the lowest concentration of amphetamine was found in a sample of hair collected from an individual with light blonde hair (Jakobsson & Kronstrand, 2014). The samples of hair used for this study were collected from participants involved in a controlled study who were given the same amount of amphetamine over a period of two weeks.

The higher accumulation of several drugs in darker hair compared with lighter hair introduces the potential for not only colour bias, but racial bias as well. Several studies have shown that, when exposed under identical conditions, different hair types incorporated different amounts of drugs (Henderson et al., 1998; Henderson et al., 1996; Joseph, Su & Cone, 1996). These studies suggest that coarse, dark hair is most likely to incorporate more drug than fine, brown or blond hair. Henderson et al. (1996) reported that, following intravenous administration of radiolabeled cocaine, Non-Caucasians incorporated 2 to 12 times more cocaine than Caucasians. In order to avoid bias, race and hair colour need to be considered when interpreting hair analysis results.

7.3 The Effect of Cosmetic Treatments

Cosmetic treatments of hair, such as straightening, perming, relaxing, dyeing and bleaching, all involve the use of chemicals that change the chemical composition of hair. This affects the stability of drugs in hair resulting in a change to the original drug concentration (Kintz, 2000). Jurado (1997) investigated the effects of cosmetic treatments by analysing samples of hair collected from individuals who had, before sample collection, treated their hair by bleaching or dyeing. Treated and untreated portions from individual strands of hair were separated and analysed using GC-MS/MS for cocaine, opiates, cannabinoids and nicotine. The results showed that the portion of hair that had been exposed to cosmetic treatment contained a lower drug concentration than the portion of untreated hair (Jurado, 1997).

In another study looking at the effect of cosmetic treatments on drug concentration, black hairs were removed from a methamphetamine addict and then treated by bleaching, perming or colouring before analysis and quantification (Takayama, 1999). It was found that, in all cases of cosmetic treatment, the concentration of methamphetamine and amphetamine decreased significantly compared to untreated hair. This decrease in drug concentration can be explained by the loss of melanin that is caused when hair undergoes cosmetic treatments, resulting in smaller amounts of drug being incorporated into the hair. The degree of damage to the hair will also affect the concentration level of drug that is found in the hair (Jurado, 1997). Additionally, the use of harsh chemicals, such as those used in most cosmetic treatments, can increase the porosity of hair causing it to become more susceptible to passive contamination (Curtis, 2008).

Therefore, despite the many advantages of using hair as a biological matrix for drug analysis, the limitations discussed above often cause the validity of hair analysis results to be questioned.

Researchers should consider these limitations as they are beneficial to avoiding the incorrect interpretation of results (Musshoff, 2007).

8. Conclusion

Hair analysis has received increased attention over the past two decades, and significant advances have been made in this field. Newer generations of GC-MS/MS and LC-MS/MS have allowed for improved sensitivity and specificity, thus providing better scientific understanding and interpretation of results (Pragst & Balikova, 2006).

In addition to improved analytical technology, the unique characteristics of hair itself make it a valuable tool for use in forensic toxicology, specifically in situations where other biological samples are unsuitable or unavailable, such as in the case of decomposed bodies. Furthermore, hair analysis results give deeper insight into details surrounding long-term drug use that is not possible through the analysis of blood or urine. As a result, the number of practical applications of hair analysis will continue to increase as research into this field progresses. Already this approach to drug analysis has been applied to some interesting cases, for example determining gestational drug exposure (Nakahara, 1999).

However, before the process of hair analysis can be fully accepted the issues surrounding external contamination as well as the differences in drug incorporation and binding in hair of different colours need to be resolved. Additionally, weak points in present hair analysis methods must be overcome by preparing reliable reference standards for interlaboratory quality control, standardising decontamination and extraction procedures and using scientifically established drug cut-off values (Pragst & Balikova, 2006).

It is evident, from literature, that the potential of hair analysis has been well recognised by a number of laboratories around the world and that the future prospects of this approach to drug analysis are widespread. However, researchers in South Africa are yet to recognise the potential that hair analysis holds. Methods need to be developed so that the field of toxicology, specifically forensic toxicology, can benefit from the practical applications that are made available by analysing hair for drugs.

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Chapter 3: Research Article

Development of a method for the screening and quantification of methamphetamine, and its major metabolite amphetamine, in hair using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Hair has recently been recognised as an important biological matrix for the detection of drugs. In this study a liquid chromatography-tandem mass spectrometry method was developed for the detection and quantification of amphetamine, methamphetamine and methylenedioxymethamphetamine in human hair. Method development involved optimising washing, extraction and chromatographic analysis conditions. Hair samples were washed in a three step process using ethanol and dichloromethane to remove external contamination. Extraction solvent made up of water, hydrochloric acid (1 M) and methanol, was added to 20 milligrams of washed hair. Hair samples were pulverized for five minutes as a part of the extraction procedure. The supernatant was analysed using high performance liquid chromatography-tandem mass spectrometry. Deuterated amphetamine was used as internal standard for quantification. Quadratic linearity with correlation coefficients greater than 0.99 was obtained for all three analytes in hair for the concentration ranges 0.63 – 20 ng/mg and 3.13 – 100 ng/mg. The method developed was used to analyse nine authentic hair samples collected either during autopsies of suspected drug-related deaths or from known drug users. The quantification of amphetamine and methamphetamine in the corresponding blood sample collected during autopsy was also determined so that hair and blood results could be compared. The results of the study show that the method developed allowed for the detection and quantification of both amphetamine and methamphetamine in authentic samples of hair. No methylenedioxymethamphetamine was detected in any of the authentic hair samples. This is the first study to date to investigate the analysis of hair for drugs within the South African context. Future research should involve the expansion and validation of the method performed.

Keywords: amphetamine, hair analysis, liquid chromatography mass spectrometry, methamphetamine

1. Introduction

Methamphetamine, commonly known as 'tik' in South Africa, is the most common primary substance of abuse in the Western Cape (SACENDU, 2014). Methamphetamine and its main metabolite, amphetamine, belong to the amphetamine class of drugs. These types of drugs are powerful stimulants of the nervous system and can cause increased alertness, insomnia, energy and self-confidence (de la Torre et al., 2004). Methylenedioxymethamphetamine (MDMA), commonly known as 'ecstasy' is a well-known amphetamine-type stimulant that is also regularly abused in South Africa and commonly associated with the rave scene (Peltzer et al., 2010). A recent study linked increased rates of crime, violence and corruption within a Cape Town community to 'tik' abuse by community members (Watt et al., 2014). The most objective manner in which to diagnose drug use is by the analysis of biological fluids (Kintz, 2004). Forensic toxicology, therefore, plays an important role in determining the details surrounding possible drug-related cases.

Although blood and urine have traditionally been used as biological matrices for the detection of drugs, such as amphetamines, hair has recently been recognised as an alternate or complementary matrix for drug analysis (Miller, Wylie & Oliver, 2008). The analysis of hair for drugs is often used to complement blood and urine testing as hair provides a large detection window for drugs (months to years) compared to blood and urine (two to four days) (Kintz, 2004). As a result blood and urine testing provide information concerning recent drug use, whilst hair analysis results provide a long-term history of drug use. Moreover, segmental analysis of hair allows for the determination of patterns of drug use including the approximate time of drug exposure (Miller, Wylie & Oliver, 2008). Other advantages of hair analysis for drugs include non-invasive sample collection that can easily be supervised thereby reducing the risk of sample tampering, and samples are easy to transport and store (Cooper, Kronstrand & Kintz, 2012).

There are, however, controversies regarding hair analysis for drugs that affect the interpretation of results. The exact mechanisms involved in the incorporation of drugs into hair are still unknown. A generally accepted model proposed by Henderson (1993) (Henderson, 1993) suggests that drugs can be incorporated into hair by at least three mechanisms namely, passive diffusion from blood capillaries into growing hair cells, from sweat and sebum and from the external environment. The possibility of external contamination in a hair sample introduces the potential for false positive results. It is therefore important that hair analysis methods include a wash procedure that is effective in removing external contamination (Kintz, 1995).

Another concern in hair testing, especially in the field of forensic toxicology, is quality assurance. In the case of blood and urine testing, calibrators and controls are easily produced by adding certain amounts of drug to a blank sample. However, hair is a solid matrix which makes it difficult to spike. Such that the addition of drugs to drug free hair does not model the process by which drugs are incorporated into the hair matrix through ingestion as the added substances lie on the surface of the hair and can easily be washed away (Sachs, 1997). To overcome this issue laboratories are using reference materials which are prepared by soaking drug free hair in solutions containing the analytes of interest for long periods of time to promote the incorporation of the analytes into the hair matrix (Welch, 2003). Reference materials are useful as they have certified values of analytes, however they are expensive and not easily available (Lee et al., 2008).

In order to assist laboratories, the Society of Hair Testing (SoHT) has published guidelines with recommendations regarding the quality assurance of hair analysis for drugs (Cooper, Kronstrand & Kintz, 2012). Further guidelines have also been recommended by the SoHT concerning the hair analysis procedure in general (Cooper, Kronstrand & Kintz, 2012). Hair analysis involves steps of washing hair to remove external contamination; drying, cutting and weighing hair samples; drug extraction; and screening and/or quantification of drugs. There is little consensus on the exact procedures to follow, and several different methodologies for the analysis of hair for drugs have been described over the past few years, including methods for the detection of amphetamine, methamphetamine and MDMA in hair (Jakobsson & Kronstrand, 2014; Tabernero et al., 2009; Chèze et al., 2007; Miyaguchi et al., 2007; Miki, Katagi & Tsuchihashi, 2003; Kintz et al., 1995).

Many different approaches are discussed in literature regarding how hair samples should be washed (Schaffer, Wang & Irving, 2002; Paulsen et al., 2001; Baumgartner & Hill, 1993). The SoHT recommends that washing procedures include steps with both organic solvents which remove surface contamination from the hair and aqueous solutions which cause the hair to swell and cause drugs from within the hair matrix to be extracted (Cooper, Kronstrand & Kintz, 2012). Additionally, there are several approaches as to how to extract drugs from hair, although, in more recent methods (Koster et al., 2014; Montesano, Johansen & Nielsen, 2014; Miyaguchi et al., 2007) hair is pulverized during extraction. Research suggests that pulverizing the hair facilitates the extraction of drugs from the hair matrix by destroying the physical structure of the hair (Miyaguchi et al., 2007).

Screening and quantification of drugs is performed using analytical techniques such as liquid or gas chromatography coupled to mass spectrometry. The use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), however, is becoming more common in drug analysis as, unlike gas chromatography, there is no chemical derivatization step which can complicate the analysis (Pragst & Balikova, 2006). Hair analysis methods that allow for improved sensitivity and selectivity are continuously being developed. The recent introduction of ultra-high performance chromatography (UPLC) has expanded the number of analytes that can be screened for in a single chromatographic run to more than 100 (Montesano, Johansen & Nielsen, 2014).

Whilst research into the field of hair analysis for drugs is rapidly advancing in many parts of the world, this is not the case in South Africa. Before this study hair samples were not collected during routine autopsies for toxicological analysis and no analysis of hair for drugs was being performed. The purpose of this study was to initiate the collection of hair samples during suspected drug-related autopsies and to develop a method to detect and quantify amphetamine, methamphetamine and MDMA in authentic hair samples using a LC-MS/MS method. The additional development of a method for the extraction and analysis of these drugs in blood allowed for blood and hair results from the same forensic autopsy case to be compared. This study presents results that will lay the foundation for future hair analysis for drugs in South Africa.

2. Materials and Methods

2.1 Chemicals and reagents

A standard solution, a mixture of six amines, namely amphetamine, methamphetamine, phentermine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA), at 250 µg/ml in methanol, was purchased from Cerilliant (Round Rock, Texas, USA). Working solutions of 10 and 100 µg/ml were prepared in distilled water. The deuterated internal standard, amphetamine-D_5, at 100 µg/ml in methanol, was also purchased from Cerilliant (Round Rock, Texas, USA). A working solution of 10 µg/ml was prepared in distilled water. Solutions were stored at – 80 °C when not in use. The purity of the reference compounds was > 99 %. The structures of the amines relevant to this study are shown in Figure 1. Acetonitrile, methanol and ethanol (gradient grade) as well as ammonium acetate and dichloromethane were purchased from Merck (Darmstadt,

Germany). Formic acid was purchased from Fluka Analytical (Sigma Aldrich, USA). Water used for the HPLC mobile phase was ultrapurified and filtered through a Synergy Water Purification System (Millipore, France).

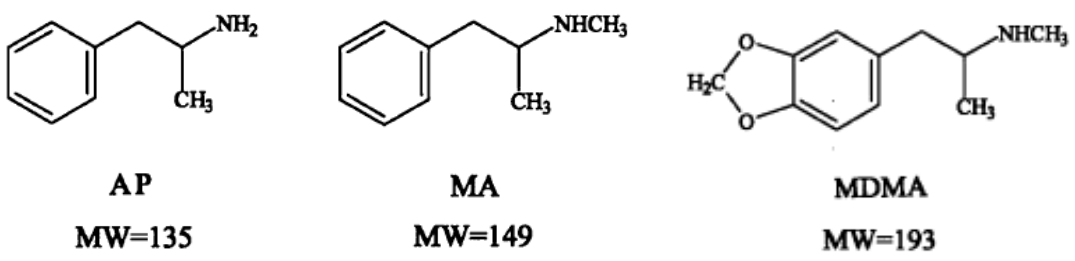

Figure 1: Chemical structures and molecular weights (MW) of amphetamine (AP), methamphetamine (MA) and MDMA (Tabernero et al., 2009).

2.2 Hair sample collection

Before the collection of any hair samples occurred, three repositories were registered to allow for the recording, storage and use of all hair samples collected. The repositories allowed for the collection of blank or drug-free hair; hair samples from drug users and forensic hair samples collected during autopsy. Drug-free hair samples, to be used in the preparation of calibration curves, were collected from a single volunteer with no history of drug abuse. Amphetamine positive hair samples (n=4) were collected from volunteers, attending a drug counselling centre, who had admitted to amphetamine use, within the three months prior to collection. Hair samples were cut from the posterior vertex region of the head as close to the scalp as possible and placed into labelled envelopes.

Forensic hair samples (n=5) were collected by pathologists during routine autopsies of suspected drug-related deaths. Hair samples were cut, at the start of an autopsy, from the posterior vertex region of the head as close to the scalp as possible. In one case, however, the sample of hair was cut from the pubic region. After collection, the hair was placed into envelopes that were sealed and labelled with a sticker recording all of the necessary information such as the date, case number, site of hair removal and hair colour. To ensure that chain of custody was maintained at all times, the hair samples were transported along with the blood and urine samples routinely

collected for toxicological analysis to the laboratory where they were signed for. All hair samples were stored in sealed envelopes at room temperature.

Ethics approval was granted by the Human Research Ethics Committee (HREC REF: 257/2014).

2.3 HPLC-MS/MS conditions

HPLC-MS/MS analysis was performed on a Shimadzu Prominence High Performance Liquid Chromatography System (Shimadzu Corporation, Kyoto, Japan) coupled to an API 3200 Q trap Mass Spectrometer (ABSCIEX, Toronto, Canada). Chromatographic separation was achieved with a Kinetex C18 column (50 mm x 3 mm, 2,6 µm) (Phenomenex, California, USA) at 40 °C with mobile phase A as 10 mM ammonium acetate with 0.1 % formic acid and mobile phase B as methanol with 10 mM ammonium acetate and 0.1 % formic acid. The system was run in a linear gradient from 5 % organic phase to 95 % organic phase in 2 minutes. The organic phase was then decreased back to 5 % from 2.10 to 4.90 minutes. The total run time was 5 minutes at a constant flow of 0.3 ml/min.

The mass spectrometer was equipped with an electrospray ionization probe, and ionization was performed in positive mode. The ion spray voltage was set to 5000 V and the source temperature was 450 °C. Direct infusions of reference solution at 10 µg/ml diluted in 50 % mobile phase A and 50 % mobile phase B were performed and from the spectra obtained, precursor ions were selected and fragmented. Two MRM transitions were chosen – the most abundant was used as the quantifier and the other as the qualifier. The MRM transitions, declustering potentials, collision energies and collision cell exit potentials are described in Table 1. Flow injection analysis was used to optimise all of the mass spectrometry parameters. Chromatograms were analysed with Analyst 1.6.2 (ABSCIEX, Toronto, Canada).

2.4 Blood sample preparation

Blood samples were prepared by adding 50 µl of water to 100 µl of blood. After vortexing briefly 500 µl acetonitrile (with 100 ng/ml internal standard) was added. Samples were vortexed, and sonciated for 10 minutes before centrifuging at 13 000 rpm for five minutes. The supernatant was removed, diluted 1:1 with water and transferred into glass vials. Ten microliters were injected into the HPLC-MS/MS system for analysis.

Table 1: Transitions and MS/MS conditions for each analyte and internal standard

Analyte	Q1 (m/z)	Q3 (m/z)	Voltages (V)		
			DP	CE	CXP
Amphetamine	136	91	21	25	2
		119	21	25	2
Methamphetamine	150	91	36	27	2
		65	36	51	2
MDMA	194	163	36	15	4
		135	36	33	2
Amphetamine-D_5	141	124	26	13	4
		93	26	25	2

Q1 – precursor ion, Q3 – product ion, m/z – mass to charge ratio,
DP – declustering potential, CE – collision energy, CXP - collision cell exit potential

2.5 Hair sample preparation

Hair samples were washed once with ethanol for five minutes, and twice with dichloromethane for five minutes each to remove any external contamination. The last wash was analysed to check if any of the analytes of interest were detected. The washed hair was air-dried and added to 2 ml microtubes with four steel balls (2.4 mm) (Omni International, Gerogia, USA) for pulverization. Hair was pulverized using the Omni Bead Ruptor 24 (Omni International, Gerogia, USA) for two cycles of 50 seconds each at 6 $m.s^{-1}$ after which 20 mg of pulverized hair was weighed and added to 2 ml microtubes. Two steel balls were added along with 1 ml extraction solvent (100 µl methanol, 65 µl HCl (1 M) and 835 µl water) and 10 µl internal standard (10 µg/ml). Samples were vortexed briefly and then pulverized for five minutes at 5 $m.s^{-1}$. After a centrifugation step the supernatant was removed and centrifuged again at 13 000 rpm for five minutes. Ten microliters were injected into the HPLC-MS/MS system for analysis.

2.6 Preparation of calibration curves and quality controls

Calibration curves were prepared by spiking samples of blank blood and hair with the highest concentration of standard in each range and serial diluting the samples down to the lowest concentration in the range. Blood curves were established in a concentration range of 15.6 – 1000 ng/ml with seven calibration levels: 15.6, 31.2, 62.5, 125, 250, 500 and 1000 ng/ml. After the dilutions were made, the blood calibrators were prepared as described above in 2.4.

Hair curves were established in two concentration ranges: 0.63 – 20 ng/mg and 3.13 – 100 ng/mg with six calibration levels each: 0.63, 1.25, 2.5, 5, 10 and 20 ng/mg and 3.13, 6.25, 12.5,

25, 50 and 100 ng/mg. Blank hair was washed according to the procedure described above and 20 mg of pulverized hair was added to six microtubes for each concentration range. In separate tubes, extraction solvent was prepared and spiked with the highest concentrations of standard in each range: 20 ng/mg (400 ng/ml) and 100 ng/mg (2000 ng/ml) and serial diluted down to the lowest. One millilitre of each of the dilutions was added to the microtubes containing 20 mg hair. Internal standard was added to each of the tubes (10 µl of 10 µg/ml solution) and the hair was pulverized and centrifuged according to the extraction procedure described in 2.5 above. Replicates (n=2) at each calibration level were analysed over three days for blood and two days for hair. Calibration curves were obtained by plotting the peak area of analytes to internal standard versus the analyte concentration to internal standard using a weighted (1/x) quadratic regression model.

Low, medium and high quality controls were prepared in blood by spiking blank blood with 25, 400 and 800 ng/ml of standard respectively. These quality controls were used to test the stability of the analytes in blood at 4 °C and room temperature (25 °C). Low, medium and high controls, in duplicate, were kept at these temperatures for one week. After one week the quality controls were prepared, along with freshly spiked quality controls, according to the sample preparation procedure described in 2.4 above. The quality controls were run with a calibration curve and peak areas were compared in order to determine stability of the analytes.

3. Results and Discussion

3.1 HPLC-MS/MS method development

The chromatographic separation seen in Figures 2 and 3 was achieved by changing various conditions (flow rate, gradient, mobile phases and column) before determining the final method to be used. The initial flow rate was set at 0.5 ml/min, with a steep gradient of 2 % organic phase increasing to 95 % in the first two minutes of the run. The total run time was five minutes. A better separation of the peaks was achieved by decreasing the flow rate to 0.3 ml/min and changing the gradient from 2 % organic phase to 5 %. Two different columns were used during the method development stages; an Allure PFP Propyl (50 mm x 2.1 mm; 5 µm) (Restek, Pennsylvania, USA) and Kinetex C18 (50 mm x 3 mm; 2,6 µm) (Phenomenex, California, USA) column. Although the Allure PFP Propyl column was initially selected, the Kinetex C18 column was chosen to be used in the final method. Preliminary results showed that the Kinetex C18 column provided better resolution.

Various mobile phase combinations were investigated with each of the two columns in order to achieve satisfactory peak shapes and separation. All three analytes eluted just after three minutes and challenges developed as one of the analytes (methamphetamine) (Figure 3.b) split into two peaks. Attempts were made either to join the peaks or to try and separate them sufficiently. In the beginning stages of the method development 10 mM ammonium acetate (mobile phase A) and 50:50 acetonitrile:methanol (mobile phase B) were used. These mobile phases, however, resulted in poor resolution of the methamphetamine peak, which appeared as a doublet. 0.1 % Formic acid (mobile phase A) and acetonitrile (mobile phase B) also resulted in unfavourable peak shapes and poor resolution. By using methanol (mobile phase B) instead of acetonitrile, the resolution of the peaks improved, and the two methamphetamine peaks separated. Adding 10 mM ammonium acetate and 0.1 % formic acid to the methanol resulted in peaks that were more symmetrical.

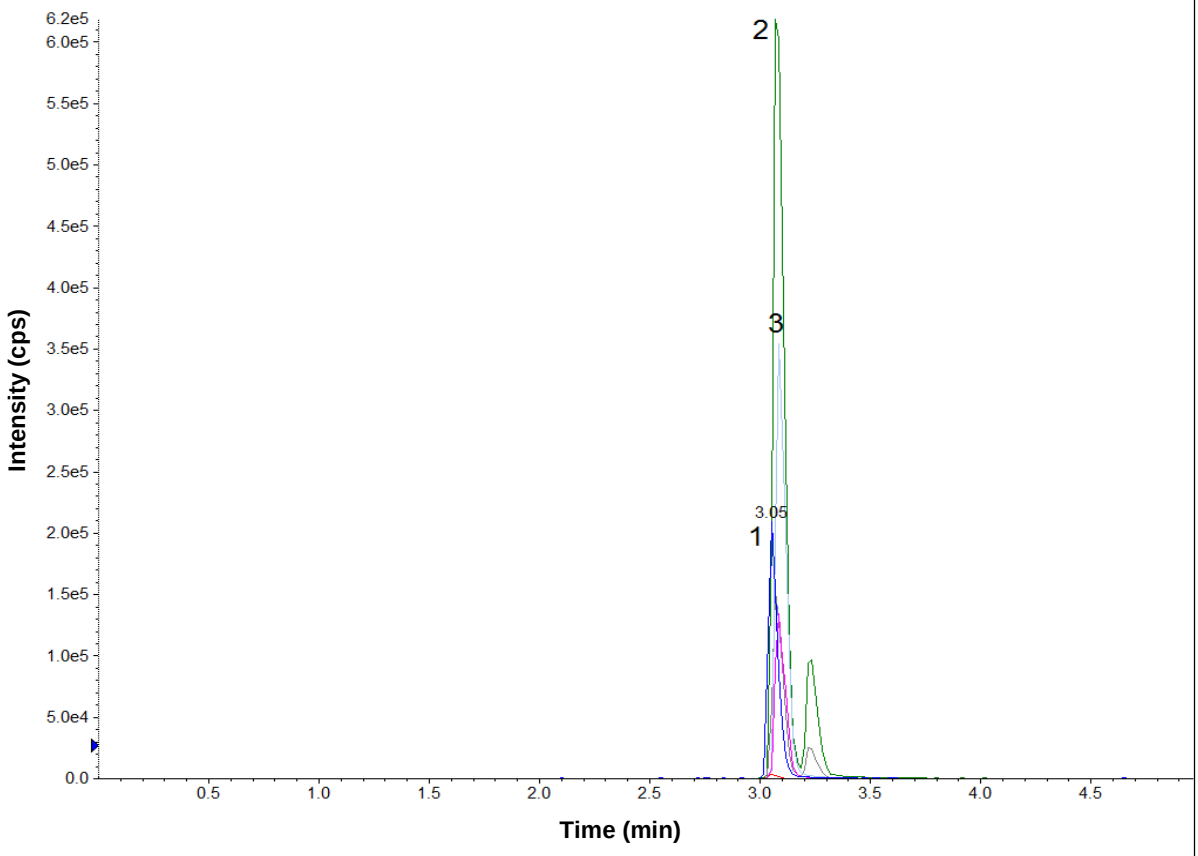

Figure 2: Chromatogram of 100 ng/ml standard in water. The quantifier ions for each analyte are numbered: **1** – Amphetamine; **2** – Methamphetamine; **3** – MDMA.

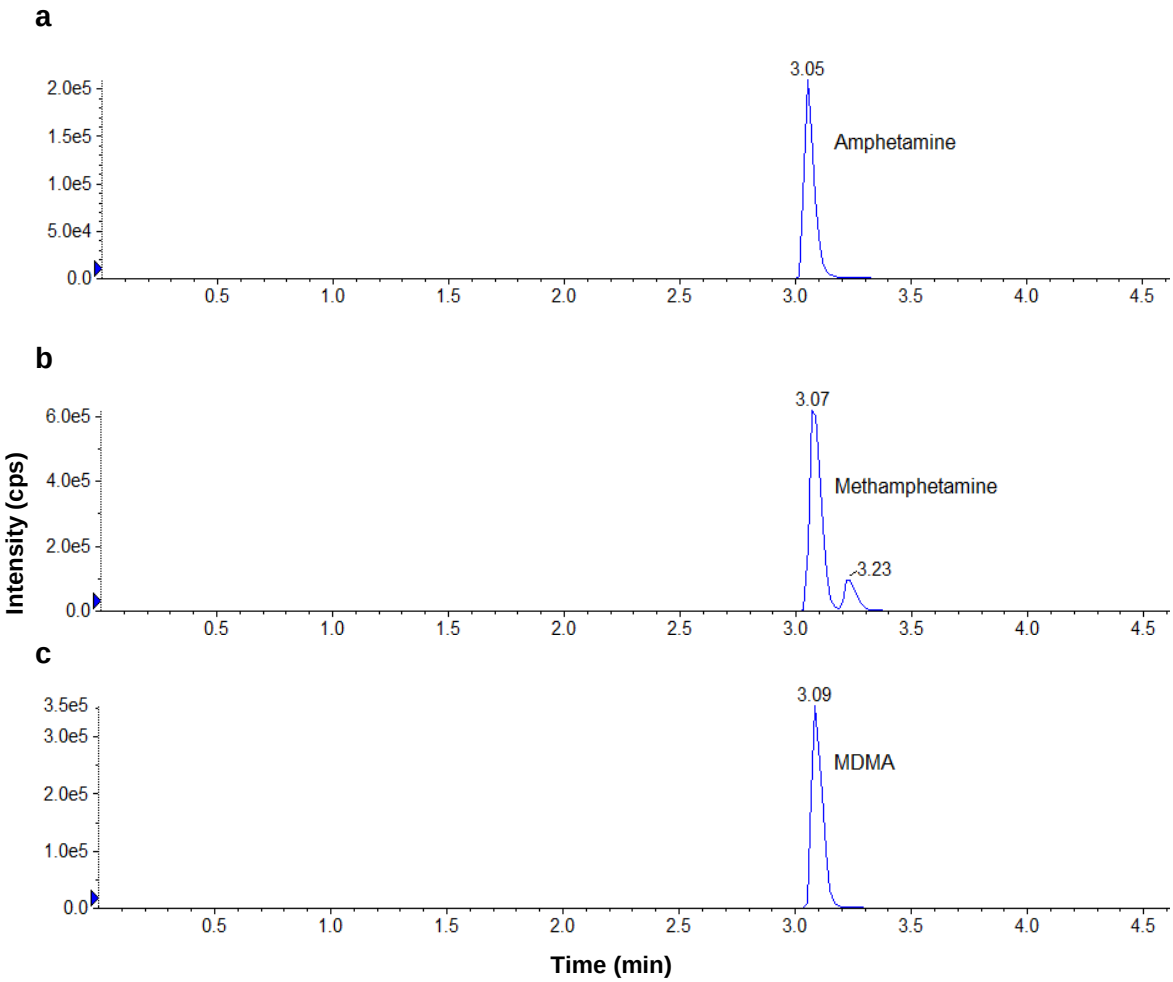

Figure 3: Chromatograms of each analyte extracted from the chromatogram shown in Figure 2. Only the quantifier ions are shown. **a:** Amphetamine (Rt = 3.05 min); **b**: Methamphetamine (Rt = 3.07 min); **c**: MDMA (Rt = 3.09).

3.2 Blood analysis

3.2.1 Calibration curves

In order to quantify amphetamine positive blood samples, calibration curves were established in duplicate at each concentration in the range 15.6 – 1000 ng/ml (Figure 4). Curves were run over three days to show reproducibility. The correlation coefficients of the calibration curves were greater than 0.99 for all of the analytes. The lower limit of quantification (LLOQ) was set as 15.6 ng/ml. This value was limited by the sensitivity of HPLC-MS/MS instrumentation available for the study.

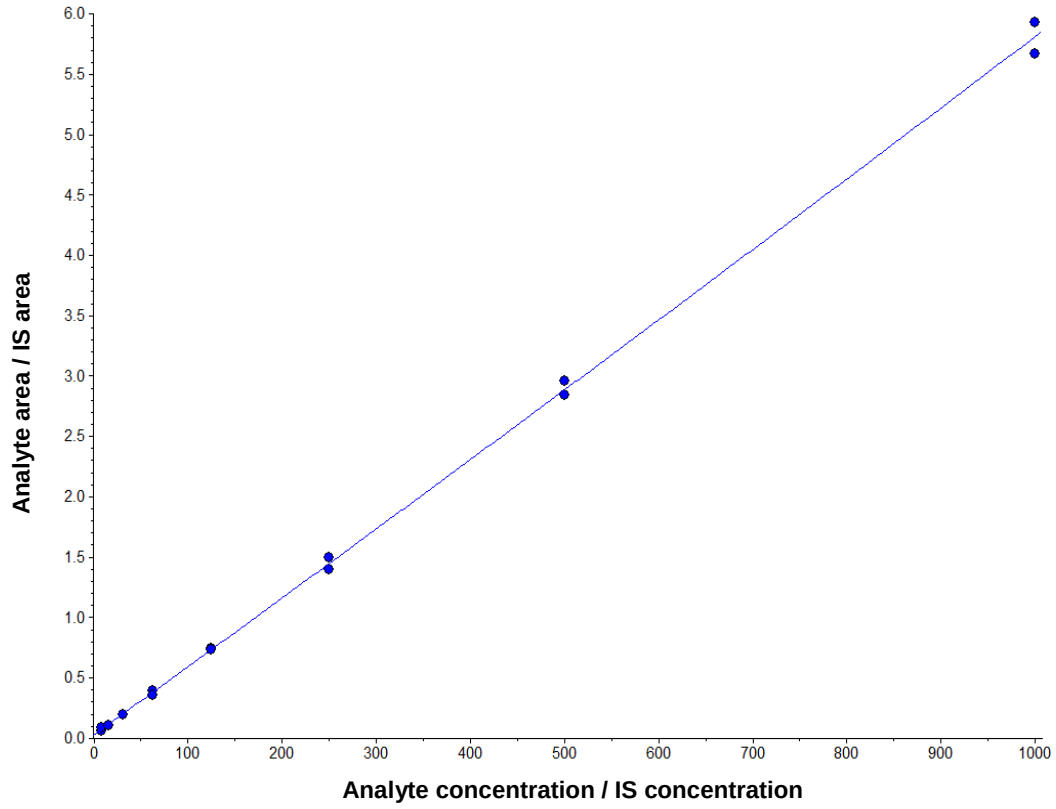

Figure 4: Amphetamine calibration curve established in blood for the range 15.6 – 1000 ng/ml representing a correlation coefficient of 0.99.

3.2.2 Stability

The stability of each of the analytes in blood was investigated by leaving low, middle and high quality controls (25, 400, 800 ng/ml respectively), in duplicate, on the bench in the laboratory and in the 4 °C refrigerator for one week. The temperature in the laboratory is maintained at 25 °C. The average peak areas for each of these quality controls were compared to the average peak areas obtained from freshly prepared quality controls for each of the analytes (Figure 5). The average difference between peak areas of fresh quality controls and quality controls kept at 4 °C was five percent. The largest difference between peak areas was calculated for the low quality controls that were kept at room temperature (21 %). The results indicate that whilst there is a greater difference in the peak areas of the analytes after a week at room temperature, they are, however, relatively stable at 4 °C.

Forensic blood samples are stored in the laboratory in the 4 °C refrigerator and, therefore, this experiment was conducted to show that the analytes are stable under these conditions. More long term experiments need to be conducted in order to test the stability of the analytes after

longer periods of time as in South Africa forensic samples are often stored at 4 °C for extended periods of time before analysis.

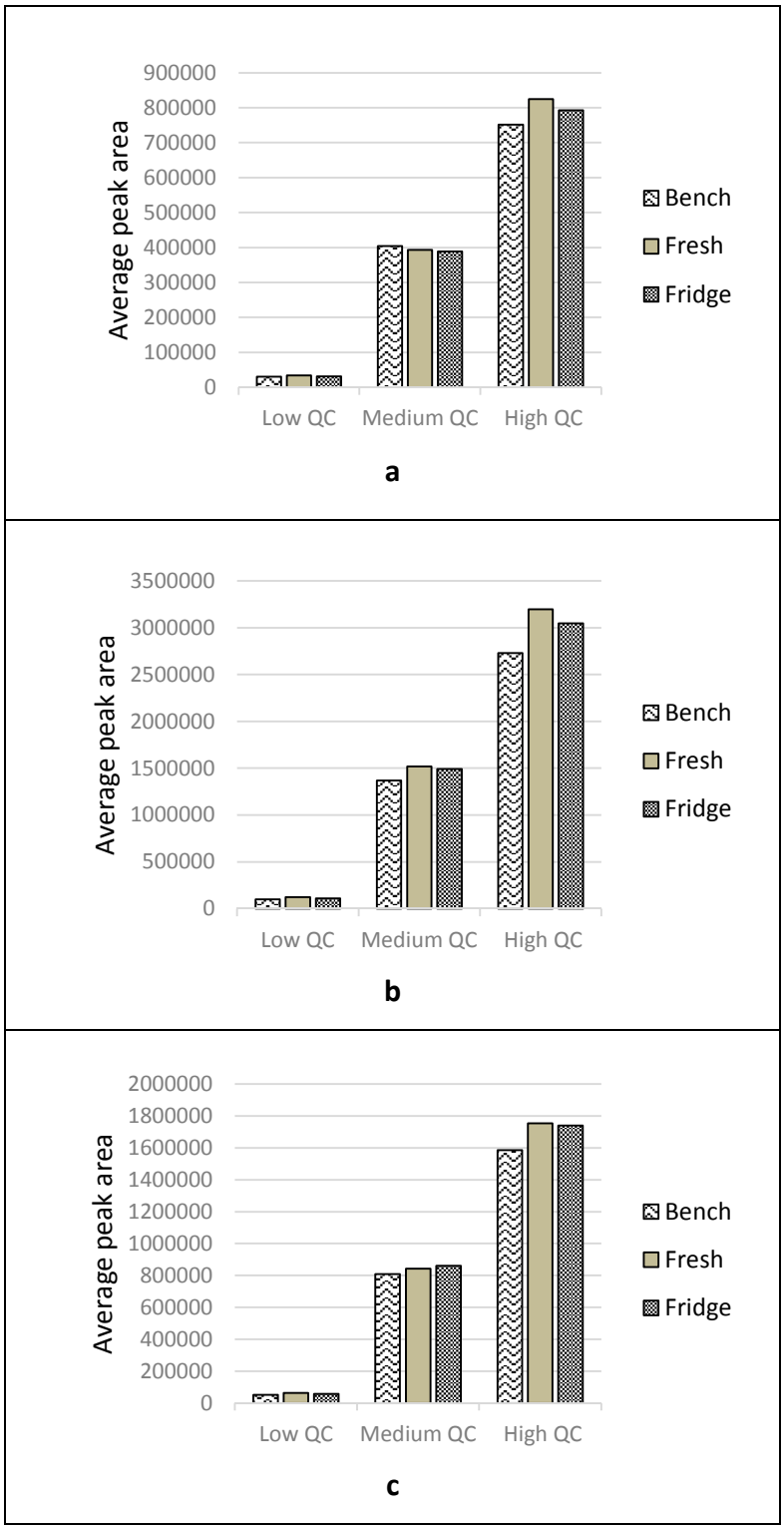

Figure 5: Bar graphs showing a comparison of the average peak areas for low, medium and high quality controls (QC) freshly prepared or prepared after one week on the bench at room temperature (~ 25 °C) or in the fridge (4 °C). These graphs show the stability of the analytes amphetamine (**a**), methamphetamine (**b**) and MDMA (**c**) under these conditions.

3.3 Hair analysis

3.3.1 Wash procedure

Three different wash procedures, as described in literature, were carried out on a sample of hair collected from a known drug user and subsequently shown to be positive for amphetamine and methamphetamine. The three procedures followed are outlined in Table 2.

Table 2: Outline of three wash procedures (1,2 and 3) carried out on amphetamine positive hair

	Solvents	**Time (min)**	**Repeats**	**Reference**
1	1. ethanol	5	1	(Chèze et al., 2007)
	2. dichloromethane	5	2	
2	1. 0.1 % SDS	3	entire procedure repeated three times	(Miyaguchi et al., 2007)
	2. distilled water	1		
	3. methanol	1		
3	1. 2-propanol	15	1	(Hegstad et al., 2008)
	2. 0.01 M phosphate buffer (pH 6)	15	3	
	3. 2- propanol	15	1	

SDS – sodium dodecyl sulphate

A sample from each wash step was analysed using the HPLC-MS/MS method developed. The chromatograms obtained were compared with regards to peaks observed. Methamphetamine peaks were observed in the wash steps of procedures two and three. According to literature, aqueous solutions and methanol cause the hair to swell and result in the extraction of drugs from the hair matrix (Cooper, Kronstrand & Kintz, 2012). Both wash procedures two and three contain aqueous solutions and/or methanol, and it is likely that the methamphetamine peaks observed were as a result of methamphetamine being extracted. It was therefore decided to use wash procedure one in this study.

3.3.2 Optimisation of hair extraction

The pulverization extraction procedure described by Miyaguchi et al. (2007) (Miyaguchi et al., 2007) was used as the starting point for the development of this drug extraction method. This was one of the first publications to report an extraction procedure involving pulverization. Initial experiments were done to determine the extent to which hair was pulverized after using a different number of steel balls and pulverizing the hair for different periods of time. Hair was fully pulverized when there were no intact strands of hair remaining. From these experiments it was determined that after two cycles of 50 seconds each at a speed of 6 $m.s^{-1}$ using four steel

balls the hair was fully pulverized (Figure 6). Hair was pulverized according to this method after washing and prior to extraction as the pulverized hair could be more accurately weighed. The method developed therefore involved two pulverization steps - after washing and during extraction.

Figure 6: A 20 mg sample of blank hair before (left) and after (right) pulverization. Hair was pulverized with four steel balls at 6 $m.s^{-1}$ for two cycles of 50 seconds each.

Amphetamine positive hair collected from a known drug user was washed using wash procedure one and used in the extraction experiments. Various modifications were made to the method previously described by Miyaguchi et al. (2007) (Miyaguchi et al., 2007) in order to develop a method to be used in this study. In the method previously described, two mg of hair was used for the extraction. Due to uncertainties regarding the sensitivity of the method as well as the amount of drug present in the hair samples, 20 mg of hair was used for the purposes of this study.

In the method described by Miyaguchi et al. (2007) (Miyaguchi et al., 2007) acetonitrile and trifluoroacetic acid were used in the extraction solvent. However, acidic methanol has previously been reported to be a good extraction solvent for the extraction of amphetamines from hair (Nakahara, 1995). Therefore, the various extraction solvents investigated consisted of water and either methanol or acetonitrile and either 1 M hydrochloric acid or 1 M trifluoroacetic acid. Other extraction conditions such as the amount of acid added to the extraction solvent, the time period for which the samples were pulverized and the inclusion of an ultrasonification or incubation step were also investigated. The results of the above experiments showed no

significant difference between the extraction methods investigated. It was therefore decided to exclude an incubation or ultrasonfication step as these proved unnecessary and increased the time of the extraction procedure. Additionally, methanol and hydrochloric acid were used in the final extraction procedure according to the literature (Nakahara, 1995). Two centrifugation steps were introduced rather than filtering the supernatant before injection into the HPLC system, although this proved to be time consuming and this step needs to be reconsidered. The determination of extraction recoveries of drugs in hair was not attempted due to the time constraints of the study as well as the limitations associated with the spiking of hair that make the determination of extraction recoveries difficult.

3.3.3 Calibration curves

Calibration curves were established by spiking blank hair with varying concentrations of standard. Figures 7.a-c show the chromatograms of a blank sample of hair spiked with a standard containing all three analytes at 5 ng/mg. Figure 7.d shows the chromatogram of the deuterated internal standard, amphetamine-D_5, added to the spiked hair before extraction.

Preliminary extraction results showed the amount of amphetamine to be much lower than methamphetamine and it was therefore necessary to establish two curves to include a wider concentration range. Curves were in the ranges of 0.63 – 20 ng/mg and 3.13 – 100 ng/mg. The LLOQ for the method was set at 0.63 ng/mg. Figure 8 shows an amphetamine calibration curve established over the range 0.63 – 20 ng/mg. The correlation coefficients for both of the calibration curves were greater than 0.99 for all of the analytes.

3.3.4 Analysis of authentic hair samples

The HPLC-MS/MS and extraction methods developed were verified by analysing authentic samples of hair for amphetamines. Hair samples were collected from known amphetamine users attending a drug counselling centre and forensic samples were collected during autopsies of suspected drug-related deaths. All of the hair samples, except for FS 4, were collected from the head and hair was cut from the vertex region as close to the scalp as possible. FS 4 was collected from the pubic region. An extract from the third wash step was analysed for each of the cases to indicate the presence of any external contamination that could affect the interpretation of results.

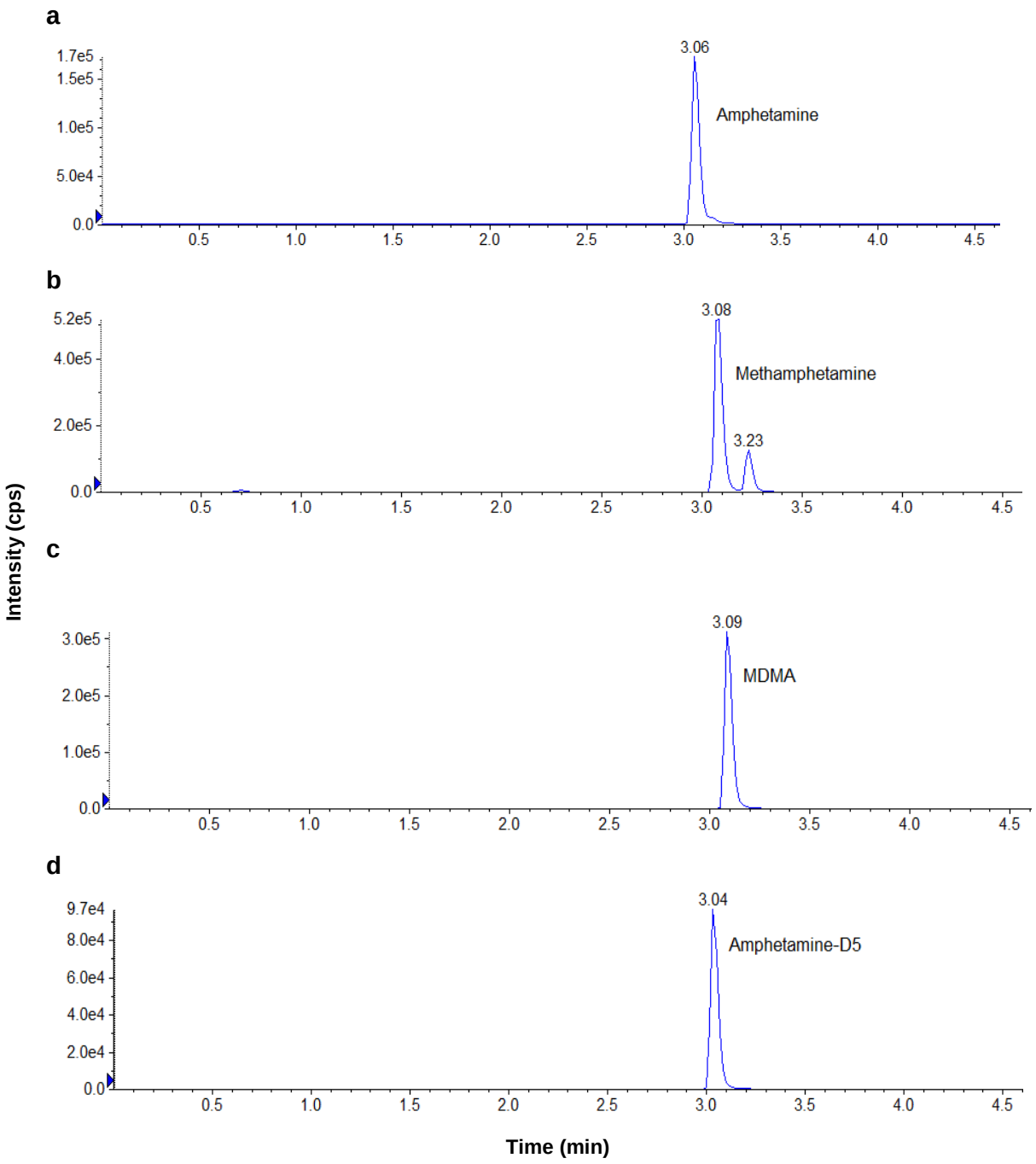

Figure 7: Chromatograms of a blank hair sample spiked with standard (5 ng/mg), containing all three analytes, and internal standard. Only the quantifier ions are shown. **a:** Amphetamine (Rt = 3.06 min); **b**: Methamphetamine (Rt = 3.08 min); **c**: MDMA (Rt = 3.09); **d**: Amphetamine-D_5 (Rt = 3.04 min).

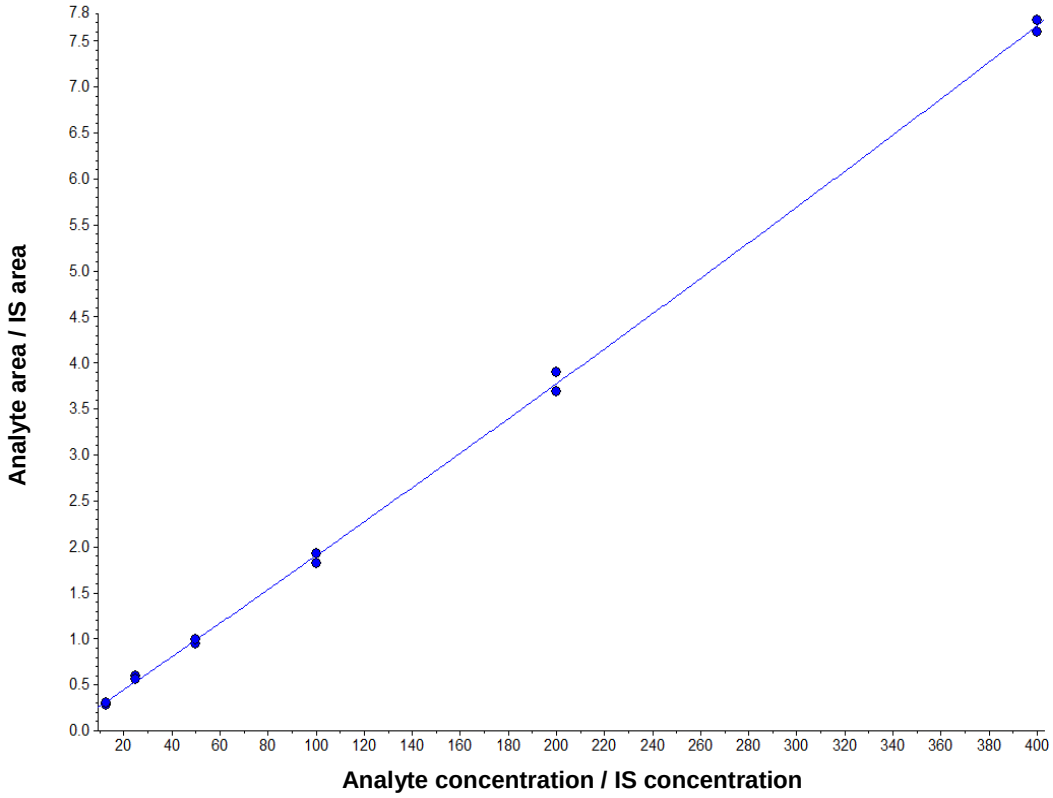

Figure 8: Amphetamine calibration curve established in hair for the range 0.63 - 20 ng/mg representing a correlation coefficient of 0.99.

a. Drug Users

The results from the analysis and quantification of these hair samples were useful in verifying that the method developed resulted in the extraction of amphetamines. Whilst it was known that amphetamine had been abused within three months before hair was collected, it was not known how much drug was ingested or when last any drug was ingested. The results show that both amphetamine and methamphetamine were detected in all four of the samples analysed (Table 3). In two of the cases (DU 2 and DU 4) the amount of drug detected was below the LLOQ and is reported as such.

The highest amphetamine and methamphetamine concentrations were detected from DU 3. Figures 9.a+b show the amphetamine and methamphetamine peaks recorded from the chromatogram obtained after the analysis. The methamphetamine peak presented as one peak in all of the authentic samples. The chromatogram from the analysis of the third wash step is also shown (Figure 9.c) to illustrate that the washed hair did not contain any of the drugs of interest before the extraction procedure.

Table 3: Quantification results from the analysis of hair samples from four drug users

Case	Amphetamine (ng/mg)	Methamphetamine (ng/mg)
DU 1	0.86	8.1
DU 2	<LLOQ	3
DU 3	1.44	20.5
DU 4	<LLOQ	<LLOQ

DU – Drug User

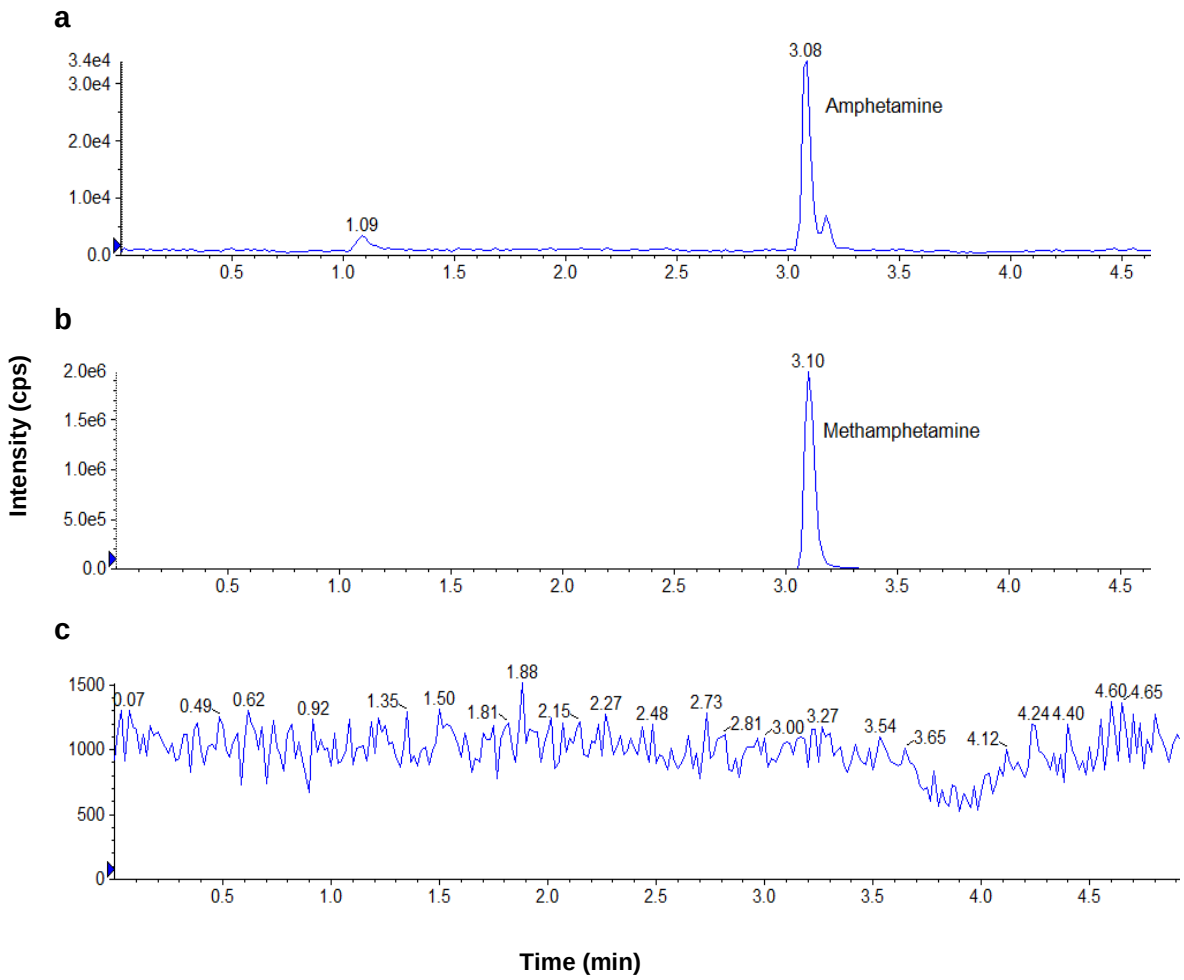

Figure 9: Chromatograms of an authentic hair sample collected from the head of a drug user (case DU 3). **a:** Amphetamine; **b**: Methamphetamine; **c:** Extract from the third wash step.

b. Forensic Samples

Forensic samples (urine, blood, bile, stomach contents, and vitreous fluid) from the autopsies of suspected drug-related deaths that are sent to the laboratory are confirmed positive or negative for the presence of drugs through a routine drug screen by HPLC-MS/MS. As a result of procedures put in place for this study, samples of hair are currently also being collected during autopsy and sent to the laboratory for drug screening. From the results of the routine drug screen, five amphetamine positive cases for which both blood and hair samples were available were chosen for analysis (Table 4).

Table 4: Quantification results from the analysis of blood and hair samples from five forensic cases

	Blood		**Hair**	
Case	**Amphetamine (ng/ml)**	**Methamphetamine (ng/ml)**	**Amphetamine (ng/mg)**	**Methamphetamine (ng/mg)**
FS 1	0	0	<LLOQ	1.33
FS 2	52.7	619	3.26	29.05
FS 3	99.3	919	0.98	12.1
FS 4	0	0	0	<LLOQ
FS 5	<LLOQ	<LLOQ	2.41	12.3

FS – Forensic Sample

In all of the forensic samples, except for FS 1 and FS 2, chromatograms of the third wash step showed that no external contamination was present. In the case of FS 1 and FS 2, however, the chromatograms recorded peaks identified as methamphetamine (Figure 10.a). The concentration of methamphetamine in these two wash step samples was calculated to be 0.18 and 1.44 ng/mg respectively. Even though the concentration of methamphetamine extracted from FS 1 and FS 2 was much greater (1.33 and 29.05 ng/mg respectively) than the concentration found in the third wash step the possibility that these results were influenced by the external contamination cannot be ignored. These two cases illustrate the importance of effectively washing hair samples to ensure that the quantification results cannot be questioned. It is likely that three wash steps may not be sufficient for all hair samples and as a result all wash steps should be analysed prior to extraction to ensure that no external contamination is present. Figures 10.b+c show the amphetamine and methamphetamine peaks from the chromatogram recorded after the analysis of the extraction of FS 2.

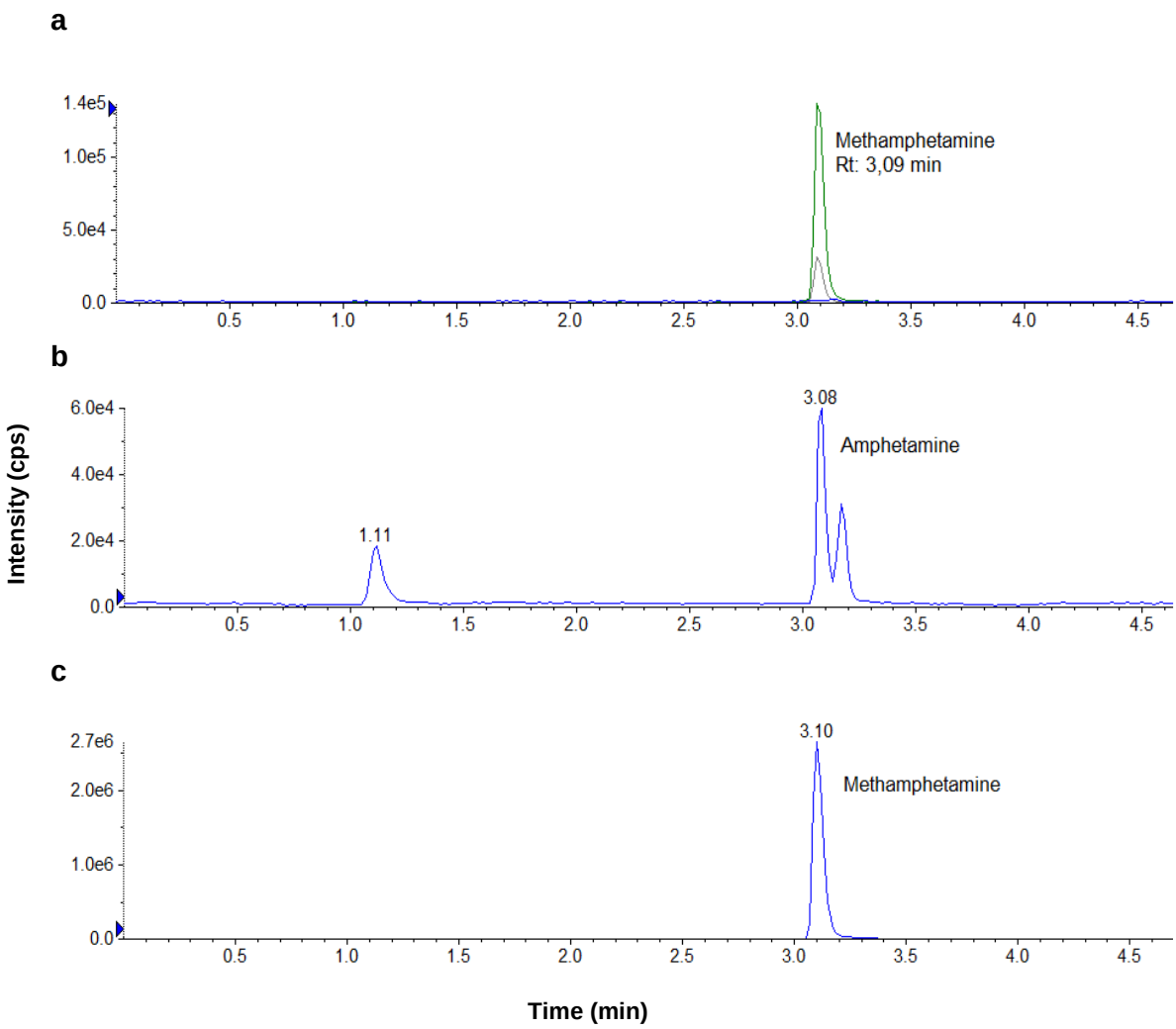

Figure 10: Chromatograms of an authentic hair sample collected during the autopsy of a suspected drug-related death (case FS 2). **a:** Extract from the third wash step indicating the presence of methamphetamine; **b and c:** Amphetamine and methamphetamine extracted from the hair sample during the extraction procedure.

High concentrations of amphetamine and methamphetamine were found in both the blood and hair samples for cases FS 2 and FS 3. In both cases the concentration of methamphetamine in the blood was well above the toxic level of 150 ng/ml (Schulz et al., 2012). Whilst there is no correlation between drug concentrations found in blood and hair, the results are useful for comparative purposes and to provide further insight into the circumstances surrounding the possible cause of death. Hair analysis results are also especially useful in cases such as FS 1 and FS 4 when drugs might have already been eliminated from biological matrices, such as blood, yet can still be detected in the hair.

The hair analysis results show that in all of the cases methamphetamine was found to be the predominant analyte. This was expected as it has been previously shown that methamphetamine has a higher incorporation rate in hair than its metabolite amphetamine (Nakahara, Takahashi & Kikura, 1995). Although the method was developed to detect and quantify amphetamine, methamphetamine and MDMA, no MDMA was detected in any of the authentic hair samples analysed.

Factors known to influence hair analysis results such as hair colour, race and chemical treatment were not considered when interpreting these results. Additionally, as extraction recoveries were not determined, it cannot be assumed that the concentrations reported in Tables 2 and 3 are representative of the total amount of drug present in the hair samples.

4. Conclusion

Washing, extraction and HPLC-MS/MS methods were developed and verified for the detection and quantification of amphetamine and methamphetamine in hair. Spiked hair samples are not a good representation of authentic hair samples concerning drug incorporation into the hair matrix. It is therefore important to verify hair analysis methods using authentic samples. Authentic blood and hair samples from five autopsy cases were analysed using the methods developed in this study. The results show that hair analysis for drugs has the potential to provide additional information regarding a case which may give further insight into circumstances surrounding the possible cause of death. The future inclusion of segmental analysis will offer invaluable information regarding an individuals history of drug abuse.

Although a method for the detection and quantification of amphetamine and methamphetamine in hair has been developed in this study, no validation parameters such as matrix effects and extraction recoveries were determined for this method. This is the first study to date to investigate the analysis of hair for drugs within in the South African context. Future research should involve the expansion and validation of the method performed.

Acknowledgements

The authors would like to thank the clinical psychologists and volunteers from the Drug Counselling Centre for their participation in the study.

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Addendum II: Instructions to Authors: Forensic Science International

Forensic Science International is a peer-reviewed, international journal for the publication of original contributions in the many different scientific disciplines comprising the forensic sciences. These fields include, but are not limited to, forensic pathology and histochemistry, toxicology (including drugs, alcohol, etc.), serology, chemistry, biochemistry, biology (including the identification of hairs and fibres), odontology, psychiatry, anthropology, the physical sciences, firearms, and document examination, as well as the many other disciplines where science and medicine interact with the law.

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References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

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There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.
If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.
Divide the article into clearly defined sections.

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Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

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Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**
• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Acknowledgements

Please provide Acknowledgements as a separate file and remove this from the manuscript. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links
Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

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Appendices

A: Permission obtained for the collection of hair during autopsies

Division of Forensic Medicine

Professor Lorna J Martin

P O Box 13914, Mowbray, 7705
Faculty of Health Sciences, Falmouth Building, Entrance 3, Level 1
Tel: +27 (0) 21 406 6412/6110 Fax: +27 (0) 21 448 1249
E-mail: lornaj.martin@uct.ac.za
Internet: www.forensicmedicine.uct.ac.za

13 February 2014

I, Lorna Jean Martin, hereby grant permission for Miss Jenna Johnston to analyse hair samples from deceased persons at Salt River Forensic Pathology Laboratory (Mortuary) which forms part of operational toxicological examination and analyses of our cases.

The research pertaining to these analyses must be anonymised by removing all identifiable patient information.

Lorna J Martin
Professor, & Head of Clinical Department

B: Information sheets and consent forms

Information Sheet: Healthy Participants

My name is Jenna Johnston and I am doing my Masters in Biomedical Forensic Science at the University of Cape Town. The research I am doing is for degree purposes and has been approved by the Human Research Ethics Committee. The title of my project is:

Development of a method for the screening and quantification of methamphetamine, and its major metabolite amphetamine, in hair using Liquid Chromatography – Tandem Mass Spectrometry

Background information about the project

The use of hair as a source of detection for drugs is rapidly increasing due to the many advantages that favour hair analysis, which can be used to determine an individual's history of drug use or abuse. In South Africa, however, hair is not currently being used in any toxicological analyses. This study aims to establish a method that will allow for the determination of the type and amount of drug/s present in a sample of hair.

Participants role

I am asking you to participate as you are healthy and do not use medication or drugs.
Samples of drug-free hair (i.e. samples of hair collected from volunteers who are not currently on any chronic medication or taking any drugs of abuse, and haven't done so within the last three months) are needed in order to enable me to develop a method to detect drugs of abuse in hair.

In order to participate in this study, you need to be healthy and not have used any drugs or medication for the last 3 months. Approximately 15 minutes of your time and a sample of your head hair will be required. Locks of hair (about a pencil thickness in total) will be cut from various places around your head. You will not be harmed in any way and there are no health risks associated with the cutting of your hair. The scissors used to cut your hair will be sterile. There are no costs involved and you will not receive any compensation for your hair sample.

The data generated from the analysis of your hair sample will be stored anonymously in a database with restricted access. You will not be identifiable in any data produced from this study and no DNA analysis will be done on the samples of hair collected. All research will be conducted in a toxicology laboratory in the Division of Pharmacology at the University of Cape Town. Your sample of hair will be stored indefinitely in the division; however, no research unrelated to this project will be done without further ethics approval.

Your participation is entirely voluntary and you can withdraw your sample of hair at any time. You are welcome to contact the researcher with any questions or concerns that you may have during the study.

Researcher: **Jenna Johnston** Cell number: 073 268 5114
Human Research Ethics Committee contact number: 021 406 6338

Informed Consent form: Healthy participants
(to be completed after reading the participation information sheet)

Development of a method for the screening and quantification of methamphetamine, and its major metabolite amphetamine, in hair using Liquid Chromatography – Tandem Mass Spectrometry

I am not on any chronic medication nor have I taken any drugs of abuse within the past three months.

The purpose and details of this study have been explained to me.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study.

I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers.

I agree to participate in this study.

Your name: _____

Your signature: _____

Researcher's signature: _____

Date: _____

Please provide your email address below if you would like to receive a copy of the final results once the study has been completed.

_____

UNIVERSITY OF CAPE TOWN
IYUNIVESITHI YASEKAPA • UNIVERSITEIT VAN KAAPSTAD

Participant Information Sheet: Drug users

My name is Jenna Johnston and I am doing my Masters in Biomedical Forensic Science at the University of Cape Town. The research I am doing is for degree purposes and has been approved by the Human Research Ethics Committee. The title of my project is:

Development of a method for the screening and quantification of methamphetamine, and its major metabolite amphetamine, in hair using Liquid Chromatography – Tandem Mass Spectrometry

Background information about the project

The use of hair as a source of detection for drugs is rapidly increasing due to the many advantages that favour hair analysis, which can be used to determine an individual's history of drug use or abuse. In South Africa, however, hair is not currently being used in any toxicological analyses. This study aims to establish a method that will allow for the determination of the type and amount of drug/s present in a sample of hair.

Participants role

You are being asked to participate, as you are either currently taking a drug of abuse, or have taken a drug of abuse in the last three months. This study has been approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee.

I am asking you to provide me with a sample/s of hair that will hopefully test positive for the drugs that you are using. These hair samples are needed in order to enable me to test the method developed to detect drugs of abuse in hair.

In order to participate in this study, approximately 15 minutes of your time and a sample of your head hair will be required. Locks of hair (about a pencil thickness in total) will be cut from various places around your head, as close to your scalp as possible. You will not be harmed in any way and there are no health risks associated with the cutting of your hair. The scissors used to cut your hair will be sterile.
There are no costs involved and you will receive a gift to the value of R50 to thank-you for your participation.

You will not be identified at any time, as your name will not be used in any of the results and your sample will be stored anonymously. The data generated from the analysis of your hair sample will also be stored anonymously in a database with restricted access requiring a password. Only I will have this password. Furthermore, you will not be identifiable in any data produced from this study and no DNA analysis will be done on the samples of hair collected. All research will be conducted in a toxicology laboratory in the Division of Pharmacology at the University of Cape Town. Your sample of hair will be stored indefinitely in the division; however, no research unrelated to this project will be done without further ethics approval.

Your participation is entirely voluntary. You are welcome to contact the researcher with any questions or concerns that you may have during the study.

Researcher: **Jenna Johnston** Cell number: 073 268 5114
Human Research Ethics Committee contact number: 021 406 6338

Informed Consent form: Drug users

(to be completed after reading the participation information sheet)

Development of a method for the screening and quantification of methamphetamine, and its major metabolite amphetamine, in hair using Liquid Chromatography – Tandem Mass Spectrometry

The purpose and details of this study have been explained to me.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study.

I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers.

I agree to participate in this study and **I am happy for my sample to be stored indefinitely, and only be used for similar research once approved by a human research ethics committee.**

Your name: _____

Your signature: _____

Please give the full name/s of any medication or drugs of abuse that you are currently taking, or have taken in the last three months:

_____

Researcher's signature: _____

Date: _____

Please provide your email address below if you would like to receive a copy of the final results once the study has been completed.

_____

C: Repository registration forms

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

23 May 2014

REF NO: R021/2014

Prof P Smith
Pharmacology
K45, OMB

Dear Prof Smith

PROJECT TITLE: ***Clinical Pharmacology Hair Repository: Blank Hair***

Thank you for your submission to the Faculty of Health Sciences Human Research Ethics Committee.

The HREC has **approved** the registration of your repository. Please confirm that HREC approval will be sought before further studies occur.

Please Note: All research, including that undertaken for a master's or doctoral degree, using registered databases, registries and repositories, requires submission as a new study. It requires an application form (FHS013) and a protocol which has undergone departmental review. The study will receive its own HREC REF number which will be linked to the main database or repository.

The registration of this database is valid until **30 May 2017.**

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

23 May 2014

REF NO: R022/2014

Prof P Smith
Pharmacology
K45, OMB

Dear Prof Smith

PROJECT TITLE: ***Clinical Pharmacology Hair Repository: Drugs Of Abuse***

Thank you for your submission to the Faculty of Health Sciences Human Research Ethics Committee.

The HREC has **approved** the registration of your repository. Please confirm that HREC approval will be sought before further studies occur.

Please Note: All research, including that undertaken for a master's or doctoral degree, using registered databases, registries and repositories, requires submission as a new study. It requires an application form (FHS013) and a protocol which has undergone departmental review. The study will receive its own HREC REF number which will be linked to the main database or repository.

The registration of this database is valid until **30 May 2017.**

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

08 May 2014

REF NO: R016/2014

Prof L Martin
Division of Forensic Medicine & Toxicology
Falmouth Building
Entrance 3
Level 1

Dear Prof Martin

PROJECT TITLE: ***Repository of Pathology Specimens from Medico-Legal Autopsies***

Thank you for your submission to the Faculty of Health Sciences Human Research Ethics Committee.

The HREC has **approved** the registration of your repository.

Please Note: All research, including that undertaken for a master's or doctoral degree, using registered databases, registries and repositories, requires submission as a new study. It requires an application form (FHS013) and a protocol which has undergone departmental review. The study will receive its own HREC REF number which will be linked to the main database or repository.

The registration of this database is valid until **30 May 2017.**

Please quote the HREC REF in all your correspondence.

Yours sincerely

pp TJB Wge

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

D: Human Research Ethics Committee approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

05 May 2014

HREC REF: 257/2014

Prof P Smith
Pharmacology
K Floor
OMB

Dear Prof Smith

PROJECT TITLE: DEVELOPMENT OF A METHOD FOR THE SCREENING AND QUANTIFICATION OF METHAMPHETAMINE, AND ITS MAJOR METABOLITE AMPHETAMINE, IN HAIR USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (MPhil Ms Jenna Johnston)

Thank you for your response letter to the Faculty of Health Sciences Human Research Ethics Committee dated 19 March 2014.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th March 2015

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/research/humanethics/forms)

We acknowledge that the MPhil student Ms Jenna Johnston will also be involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC reference no in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN ETHICS

E: Role of co-authors

Prof. P Smith

Helped develop the overall study design; gave continuous practical and administrative guidance and assistance throughout the course of the study; facilitated with the analysis of results and provided feedback during the writing up stages.

Dr. M Heyns

Assited with the initial stages of the development of the study design; assisted with the setting up of the hair collection protocol and provided administrative assistance.

F: Budget: costs incurred

Description	**Cost**
Hair collection kit (for 150 samples)	355
Compensation for volunteers (R 50 x 30)	1 500
Running of instrumentation	6 000
Equipment (e.g. HPLC column)	6 500
Reagents (e.g. HPLC solvents)	3 500
Other chemicals (e.g. reference standards, internal standard)	4 500
Additional costs: boxes for storage of hair samples	100
printing and binding of thesis	250
TOTAL	**22 705**

G: Certificates of analysis for reference and internal standards

Cerilliant
Analytical Reference Standards
a SIGMA-ALDRICH company

Certificate Quality
ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 9001
GMP/GLP

Certificate of Analysis

Amine Mixture-6

Catalog Number:	A-050
Solution Lot:	FE122911-01
Expiration Date:	January 2017
Solvent:	Methanol
Volume per Ampule:	Not less than 1 mL
Storage:	Store unopened in freezer.
Intended Use:	For R&D/ analytical purposes only. Not suitable for human or animal consumption.
Regulatory:	USDEA Exempt | Canadian TK # 61-465 **Safety:** Flammable, Poison

- Expiration Date has been established through real time stability studies.
- Ampules are overfilled to ensure a minimum 1 mL volume. We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.

Component	Chromatographic Purity	Concentration
(±)-Amphetamine	99.69%	250.1 ± 1.6 µg/mL
(±)-Methamphetamine	99.85%	250.0 ± 1.6 µg/mL
Phentermine	99.38%	250.0 ± 1.6 µg/mL
(±)-MDA	99.73%	250.0 ± 1.6 µg/mL
(±)-MDMA	99.59%	250.1 ± 1.6 µg/mL
(±)-MDEA	99.07%	250.1 ± 1.6 µg/mL

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and balance and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Concentration is calculated based on: the actual measured mass; Purity Factor of the analyte(s); measured mass of the solution; and the density of the pure diluent at 20°C.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

Traceability

- Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error.
- Concentration is verified against an independently prepared 4-point calibration curve gravimetrically prepared using balances calibrated to NIST.
- In addition, each neat material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of the COA.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

ACLASS ACCREDITED REFERENCE MATERIAL PRODUCER

Lara Sparks, Quality Assurance Director

January 18, 2012
Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78665 800-848-7837 / 512-238-9974

Standard Solution Assay Parameters

Analysis Method:	GC/FID
Column:	DB-5ms 30 m x 0.53 mm ID, 1.5 µm film thickness
Temp Program:	60°C to 260°C at 20°C/min (hold 1 min)
Injector Temp:	Cool-on-Column
Detector Temp:	325°C

Calibration Curve

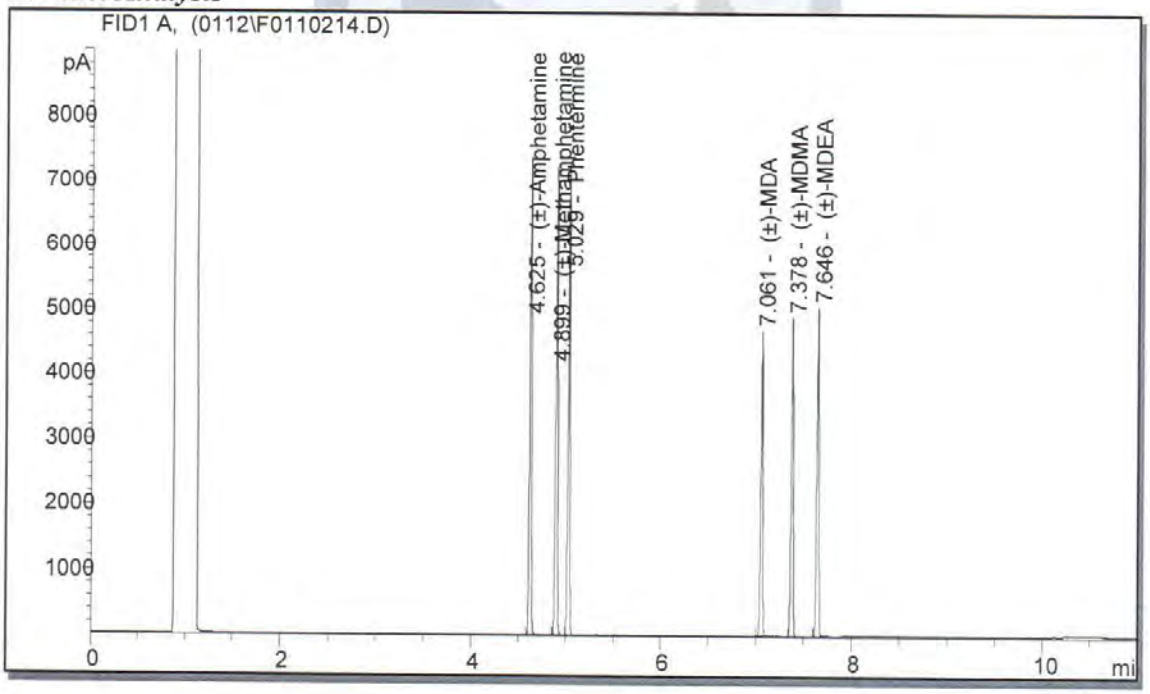
Calibration Curve:	Linear Regression
Number of Points:	4
Linearity (r):	>0.999

Solution Standard Verification and Homogeneity

Compound	Verified Concentration µg/mL	Acceptance Criteria	%RSD - Homogeneity %	Acceptance Criteria
(±)-Amphetamine	255.7	± 5%	0.1	≤ 3%
(±)-Methamphetamine	253.5	± 5%	0.1	≤ 3%
Phentermine	252.8	± 5%	0.1	≤ 3%
(±)-MDA	248.5	± 5%	0.6	≤ 3%
(±)-MDMA	247.1	± 5%	0.1	≤ 3%
(±)-MDEA	247.3	± 5%	0.1	≤ 3%

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration curve.
- Homogeneity is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. The %RSD of samples pulled from across the lot demonstrate homogeneity.
- Product is compared to prior lot and meets acceptance criteria of ± 10%.

Product Analysis



Neat Material Data

Compound	Lot Number	CAS Number	Chemical Formula	Molecular Weight
(±)-Amphetamine	FC120811-01	300-62-9	$C_9H_{13}N$	135.21
(±)-Methamphetamine	FC121410-01	4846-07-5	$C_{10}H_{15}N$	149.24
Phentermine	FC072010-01	122-09-8	$C_{10}H_{15}N$	149.24
(±)-MDA	FC041410-01	4764-17-4	$C_{10}H_{11}NO_2$	179.22
(±)-MDMA	FC011211-01	42542-10-9	$C_{11}H_{15}NO_2$	193.25
(±)-MDEA	FC032311-01	82801-81-8	$C_{12}H_{17}NO_2$	207.27

Compound	Chromatographic Purity	Residual Solvent [1]	Residual Water	Residual Inorganics	Purity Factor
(±)-Amphetamine	99.69%	None Detected	0.68%	NA	99.01%
(±)-Methamphetamine	99.85%	None Detected	0.33%	NA	99.52%
Phentermine	99.38%	None Detected	0.47%	NA	98.92%
(±)-MDA	99.73%	None Detected	0.31%	NA	99.42%
(±)-MDMA	99.59%	None Detected	0.25%	NA	99.29%
(±)-MDEA	99.07%	0.02%	0.69%	NA	98.37%

- Purity is calculated as the average of two independently performed analyses utilizing two different methods. Acceptance criteria requires the purity values to be within 0.5% of each other. A secondary purity method is used as a control but is not reported in the Certificate of Analysis.
- Purity Factor = [(100 - wt% residual solvent - wt% residual water - wt% residual inorganics) x Chromatographic Purity/100].
- Purity factor does not include adjustment for chiral and/or isotopic purity.

[1] Validated analytical method

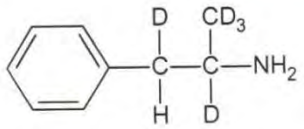
Cerilliant
Analytical Reference Standards
a SIGMA-ALDRICH company

Certificate of Analysis

(±)-Amphetamine-D_5

(±)-1-Phenyl-1,2,3,3,3-pentadeutero-2-aminopropane

Cerilliant Quality
ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 9001
GMP/GLP



Catalog Number:	A-005
Solution Lot:	FE020613-05
Expiration Date:	February 2018
Solvent:	Methanol (LC-MS Chromasolv ®)
Volume per Ampule:	Not less than 1 mL
Storage:	Store unopened in freezer.
Shipping:	Ambient. See Stability Section.
Intended Use:	For R&D/ analytical purposes only. Not suitable for human or animal consumption.
Regulatory:	USDEA Exempt | Canadian TK# 61-04 **Safety:** Flammable, Poison

- Expiration Date has been established through real time stability studies.
- Ampules are overfilled to ensure a minimum 1 mL volume can be transferred when using a 1 mL Class A volumetric pipette. We advise laboratories to quantitatively transfer desired volumes of this standard using established good laboratory practices to dilute to the desired concentration.

Component	Solution Purity	Certified Concentration
(±)-Amphetamine-D_5	99.3%	100.0 ± 0.4 µg/mL

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and balance and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Concentration is calculated based on: the actual measured mass; Purity Factor of the analyte(s); measured mass of the solution; and the density of the pure diluent at 20C.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

Solution Standard Verification and Homogeneity

Standard Solution	Lot Number	Verified Concentration (µg/mL) Actual Results	Verified Concentration (µg/mL) Acceptance Criteria	%RSD - Homogeneity Actual Results	%RSD - Homogeneity Acceptance Criteria
New Lot	FE020613-05	101.0	± 3%	0.4	≤ 3%
Previous Lot	FE072610-01	99.34	± 3%	0.3	≤ 3%

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration solution.
- Homogeneity of the New Lot is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. The % RSD of samples pulled from across the lot demonstrate homogeneity of the New Lot.
- The % RSD of the Previous Lot represents variability of the analysis performed at the time of release.

Traceability

- Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error.
- Concentration is verified against an independently prepared calibration solution gravimetrically prepared using balances calibrated to NIST.
- In addition, each neat material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of the COA.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

ACLASS ACCREDITED REFERENCE MATERIAL PRODUCER

Lara Sparks, Quality Assurance Director

March 28, 2013
Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78665 800-848-7837 / 512-238-9974

Standard Solution Assay Parameters

Analysis Method:	GC/FID
Column:	DB-5ms 30 m x 0.53 mm ID, 1.5 µm film thickness
Temp Program:	60°C to 260°C at 20°C/min hold 1 min
Injector Temp:	Cool-on-Column
Detector Temp:	325°C

Calibration Curve

Calibration Curve:	Linear Regression
Number of Points:	4
Linearity (r):	1.000

Neat Material Data

Compound Name:	(±)-Amphetamine-D_5	**Chemical Formula:**	$C_9H_8D_5N$
Compound Lot:	FC071411-01	**CAS Number:**	136765-27-0
		Molecular Weight:	140.23

Neat Material Characterization Summary

Analytical Test	Method	Results	
Primary Chromatographic Purity by GC/FID Analysis	SP10-0101	99.3%	
Secondary Chromatographic Purity by HPLC/PDA Analysis	SP10-0102	99.2%	
Identity by LC/MS Analysis	SP10-0107	Consistent with Structure	
Isotopic Purity by LC/MS SIM Analysis	SP10-0107	0.00% D_0 vs D_5	
		0.00% D_0	0.12% D_3
		0.01% D_1	1.86% D_4
		1.07% D_2	96.95% D_5
Identity by ^{1}H-NMR Analysis	USP <761>, SP10-0116	Consistent with Structure	
Residual Solvent Analysis by GC/FID Headspace	AM1087 [1]	0.10%	
Residual Water Analysis by Karl Fischer Coulometry	USP <921>, SP10-0103	2.94%	
Purity Factor		96.31%	

- Primary purity is calculated as the average of two independently performed analyses utilizing two different methods. Acceptance criteria requires the purity values to be within 0.5% of each other.
- The primary chromatographic purity value is used to calculate the Purity Factor.
- A secondary chromatographic purity method is utilized as a control.
- Purity Factor = [(100 - wt% residual solvent - wt% residual water - wt% residual inorganics) x Chromatographic Purity/100].
- Purity factor does not include adjustment for chiral and/or isotopic purity.

[1] Validated analytical method.