Partial validation for the analysis of benzoylecgonine and opiates in urine using solid phase extraction and GC/MS

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Partial validation for the analysis of benzoylecgonine and opiates in urine using solid phase extraction and GC/MS

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Dedicated to all those who helped me strive throughout my journey leading to this accomplishment

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Abstract

In recent years an increase in drug use amongst Maltese locals has been observed, particularly in relation to the use of cocaine, cannabis and new synthetic drugs. This is also supported by the increased availability of these substances. Such developments have led to the need to develop analytical methods which detect such substances in biological fluids within reasonable timeframes for legal and medical purposes. The drugs of abuse reported most frequently among frequent users and in overdose emergencies at Mater Dei Hospital (MDH), the major teaching hospital in Malta include heroin and cocaine.

This study aimed to validate techniques for the analysis of benzoylecgonine (major metabolite of cocaine) and opiates in urine, using solid phase extraction and GC/MS using commercially available test kits developed by Eureka Lab Division, used at facilities in the Department of Chemistry. Calibration curves congruent with those available with these kits were produced. After partial validation of the method was completed, this was confirmed using anonymised urine samples collected post-mortem. Any drugs/metabolites from these urine samples were extracted via Solid Phase extraction using the Eureka® kits. They were subsequently concentrated with nitrogen and analysed via GC/MS to identify and possibly measure the levels of drugs/metabolites determined in the sample. Following the relevant Ethics approval urine samples obtained from six cadavers of suspected drug overdose were analysed to validate the procedure.

All analytes were successfully detected in these six urine samples. Interday accuracy was 37.4% at low concentration and 19.49% at high concentration for

benzoylecgonine; 8.56% at low concentration and 11.19% at high concentration for cocaethylene; 40.84% at low concentration and 11.31% at high concentration for cocaine; 19.70% at low concentration and 48.90% at high concentration for morphine and 23.42% at low concentration and 11.98% at high concentration for morphine.

These results indicate that this analytical method resulted in the partial validation for the analysis of benzoylecgonine and opiates in urine using solid phase extraction and GC/MS. This analysis was undertaken with relative ease, efficacy, cost and time needed as compared to traditional liquid-liquid extraction, which facilitates its use in forensic and clinical laboratories and in rehab centres.

Contents

List of Tables

Table of Figures

List of Abbreviations

6-MAM	6-monoacetyl morphine
BEG	benzoylecgonine
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CV	coefficient of variation
DAD	Diode Array Detection
ELISA	enzyme-linked immunosorbent assay
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ENFSI	European Network of Forensic Science Institutes
ESI-MS	Electrospray ionization mass spectrometry
FID	flame ionisation detector
FREC	University of Malta Faculty of Medicine and Surgery Ethics Committee
FTIR	Fourier transform infrared spectroscopy
GC	Gas Chromatography
GC/MS	gas chromatography mass spectroscopy
GC-FID	gas chromatography flame ionisation detection
GLD	Gas Liquid Dispersion
HPLC	high performance liquid chromatography
IR	Infrared
LC	Liquid Chromatography
LC/MS	Liquid Chromatography- mass spectroscopy
LoD	Limit of detection
LoQ	limit of quantitation
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectrometry
MDH	Mater Dei Hospital
MS	Mass Spectroscopy
MS-MS	Tandem Mass Spectroscopy
NMR	nuclear magnetic resonance
SBSTFA	Selectra-Sil N,O-bis(trimethylsilyl)trifluoroacetamide molecule
SEM	scanning electron microscope
SPE	solid phase extraction
SPME	solid phase microextraction
SWGTOX	Scientific Working Group for Toxicology
TLC	Thin Layer Chromatography
TMCS	thrimethylchlorosilane
UV	Ultra violet
UV-Vis	Ultraviolet visible

Chapter 1

Introduction

1.1 Introduction

This research project considered the partial validation of analytical methods used to detect cocaine and its metabolites and opiates in urine samples. This validation was carried out with the use of commercially produced kits and took place in the University of Malta Forensic Analysis and Toxicology Laboratory. This partial validation methodology considered all the elements required for the validation of such methods established by relevant international organisations and regulatory bodies in the forensic field, such as ENFSI (European Network of Forensic Science Institutes) and SWGTOX (Scientific Working Group for Toxicology). As a result of this partial validation, the kits can be considered of the level needed to ensure results of high quality, can be confidently used in court proceedings in support of relevant court cases, and can help to detect intoxication via drugs of abuse in patients at the major teaching hospital in Malta, Mater Dei Hospital (MDH). The Courts of Malta and MDH are the two main clients of the University of Malta Forensic Analysis and Toxicology Laboratory.

A robust and validated drug detection laboratory methodology is of great significance on a national and international basis since drug use has increased greatly over the decades, particularly in relation to cocaine and cannabis use (EMCDDA, 2019). Statistics indicate that opiate use has decreased, but is still causes lethal overdoses, thus opiates are still considered as important drugs in forensic sciences. Accreditation of both medical and forensic laboratories is increasingly being requested within Europe and the rest of the world, with validation being a basic requirement for such certification (ENFSI, 2014). Even when full accreditation cannot be achieved due to

other factors, method validation increases the scientist's confidence in the obtained results and trust amongst peers. Other related professions such as the legal profession, recently gained interest in the accreditation and validation of analytical results presented in court cases. These and other factors increased the significance of this present research in the local setting.

This Chapter will provide a short review of the local drug scene in the Maltese Islands, in relation to cocaine and opiates, followed by a brief history of drug analysis methods, a description of gas chromatography – mass spectrometry apparatus and solid phase extraction methods used, and overview of the analysed substances, a literature review related to validation and accreditation, gaps in research. It will then provide the aims and objectives of this study.

1.1.1 Use of Illicit Drugs: A local perspective

Illicit drug use has drastically increased in Malta over the last years (EMCDDA, 2019). When cannabis was legalised in Malta in December 2021 (Act LXVI, Authority on the Responsible use of Cannabis Act, 2021) the country was already experiencing a sharp increase in cocaine abuse, with half of the new drug users of the year being cocaine users (National Focal Point for Drugs and Drug Addiction, 2022). This trend was confirmed with increased cocaine seizures and street level purity of the drug (National Focal Point for Drugs and Drug Addiction, 2022). Heroin use has decreased in the last 5 years, as indicated by statistics related to opioid agonist treatment (National Focal Point for Drugs and Drug Addiction, 2022), but, up to 2021, was still the primary drug

for half those under treatment for drug abuse, indicating it is still problematic locally. The heroin user population is steadily aging, with the current median age of users being 41 years and that of first use being 18 (National Focal Point for Drugs and Drug Addiction, 2022). This suggests that even if new entrants to the cohort are decreasing in number, the quantity of users is still significant at 990 users as of 2021 (National Focal Point for Drugs and Drug Addiction, 2022). This was higher than the total cocaine users under treatment, 676 individuals as of 2021 (National Focal Point for Drugs and Drug Addiction, 2022). These numbers indicate cocaine and heroin are amongst the most problematic drugs in the local scene, both numerically and by severity of the resulting addiction, with daily users having increased for both drugs in 2021 (National Focal Point for Drugs and Drug Addiction, 2022). This, sadly, resulted in increased hospitalisation due to overdose, and even fatalities. People under the influence of drugs are a danger to themselves and others, prone to accidents which can be fatal to both them and bystanders. In 2021, 273 individuals sought hospitalisation for cocaine intoxication (Marianna Calleja, 2023). Locally, 25–44-year-olds accounted for all drug induced deaths in 2021 (EMCDDA, 2023), with 33% being caused by opioids. Thus, cocaine and opioid urinalysis are of great significance to local medical and forensic sectors.

Locally, it is illegal to drive under the influence of both heroin and cocaine (Cap. 65 of the Laws of Malta), as is general possession or use (Cap.101 of The Laws of Malta). Being caught under the influence by police can lead to arrest and prosecution, and being involved in any accident while under the influence can influence court sentence,

as per the Drug dependence Act (Cap.537 of The Laws of Malta). Thus, drug analytical tests may be ordered by the Inquiring Magistrate in order to confirm whether such substances were present. This is also requested in cases where someone is found dead under unusual circumstances.

1.2 Drug analysis of illicit drugs

1.2.1 History of drug abuse and addiction

Drug use is a problem that has existed for time immemorial, with addiction to substances such as opium and coca plants (the source of heroin and cocaine respectively) being documented since the Middle Ages and prior. It was not until the last century, however, that society at large started being concerned and somewhat proactive about this malady, with the US being the pioneer of urine drug testing in the 1970's. Urine was the first fluid to be used for such purpose, as it is obtained non-invasively and with ease (Zhang, Liu et al., 2022)

In modern times, the first molecule to be tested for as implicated in accidents was alcohol, through gadgets including the drunkometer in 1938 and breathalyser in 1961, and laboratory procedures including gas chromatography in the first half of the 20th century (Christophersen, Morland et al., 2016). Drugs of abuse were soon to follow, with paper chromatographic analysis procedures being developed in the 1950's (Christophersen, Morland et al., 2016), and UV-Vis and Infrared spectrometry being introduced in the 60's (Wennig, 2002). These techniques used electromagnetic radiation to identify molecules, as different molecules absorb and reflect specific

frequencies, thus are detected as a specific wavelength by the apparatus. Thin layer chromatography is used for herbal preparations such as cannabis (Rendle, 2005). Paper chromatography and TLC (thin layer chromatography) use forces such as gravity and adhesion to separate different substances, and are used with more crude mixtures as they are not damaged as easily by impurities, but are not as specific as other techniques in identifying the substance. Significant advancements occurred in the 1970's and 80's, with Gas Chromatography and Liquid chromatography being introduced for biological fluid analysis for drugs (Jones, Morland, et al., 2019). Both techniques were combined with mass spectrometry in the 80's (Rennig, 2002). Concurrently, enzyme immunoassays emerged in the 70's, with these being used for initial screening in conjunction with GC, MS, GC/MS (gas chromatography/ mass spectroscopy) or LC/MS (Liquid Chromatography/mass spectroscopy) for confirmation since the 1990s (Christophersen, Morland et al., 2016). ELIZA (enzyme-linked immunosorbent assay) needs to be confirmed by GC/MS as they show numerous cross-reactivities, and thus produce questionable results (Yanez-Sedeno, Agui et al., 2014). A non-exhaustive list of analytical methods for drug detection is listed in Table 1.1, and discussed in Section 1.2.2.

Presently GC/MS is considered by many as the gold standard of forensic toxicology, however LC/MS has gained popularity (Perez, Knapp et al., 2016). The trust scientists put in these techniques is due to the high precision and accuracy achievable with both, the techniques functioning both to separate the analyte from other similar compounds, identification via the retention time of the chromatography apparatus

and quantitation via the Mass spectrometer. This way GC/MS functions as two systems combined into one.

Table 1.1 a: Non exhaustive list of different separation and identification techniques, and oldest paper referencing the use of the method

Analytical method	Brief description	Oldest retrieved paper	Date published
Thin Layer Chromatography (TLC)	Solid medium arranged in a sheet-like manner used in conjunction with solvent to separate a mixture. Can be used for identification via retention factors.	Improved detection and identification of basic drugs extracted from tissue with TLC.	1982
Column chromatography	Solid medium packed in a column used to separate mixtures within solvents. Identification may be possible like TLC.	Liquid-solid extraction of lyophilized biological material for forensic analysis. I. Application to urine samples for detection of drugs of abuse.	1971
Infrared spectroscopy	Detection of molecules/fragments by excitation to IR radiation and detection of the released frequencies.	A multivariate analysis of the infrared spectra of drugs of abuse	1980
Flame ionisation detection (FID)	Molecules are heated to very high temperatures to reach an excited state, then the released heat is measured.	A review of some GLD-FID derivatization techniques found useful in forensic toxicology.	1974
Diode array detection (DAD)	Light beams are directed at the sample, the reflected wavelengths are measured by the detector to determine the substance.	Analysis of opiates, cocaine and metabolites in urine by high-performance liquid chromatography with diode array detection (HPLC-DAD)	2005
Ultra Violet absorption (UVA)	UV light is shone through the sample and the emergent light is measured for frequency and intensity to detect functional groups.	Identification of drugs and other toxic compounds from their ultraviolet spectra. Part III: Ultraviolet absorption properties of 22 structural groups	1976

Table 1.1 b: Non exhaustive list of different separation and identification techniques, and oldest paper referencing the use of the method

Liquid chromatography mass spectrometry (LC-MS)	Liquid chromatography consists of a column packed with solid through which a sample mixed in solvent is passed to separate the components of the mixture, then mass spectroscopy is used to identify the molecules.	Identification of anhydroecgonine ethyl ester in the urine of a drug overdose victim	2005
Liquid chromatography tandem mass spectroscopy (LC-MS/MS)	Similar to LC-MS, but two rounds of ionisation are performed with MS, giving more detailed readings.	Determination of illicit drugs and their metabolites in human urine by liquid chromatography tandem mass spectrometry including relative ion intensity criterion	2007
High performance liquid chromatography (HPLC)	Similar to Liquid chromatography but works with smaller volumes and uses shorter columns to reduce processing time. Can be combined with MS.	Determination of cocaine and benzoylecgonine in human amniotic fluid via high flow solid-phase extraction columns and HPLC	1992
Capillary electrophoresis	A capillary tube connected to a high voltage power supply, which when switched on upon sample entry via an introduction system into the tube, separates the components of the mixture which reach the detector for identification via computer.	Capillary electrophoresis for the investigation of illicit drugs in hair: determination of cocaine and morphine	1993

Table 1.1 c: Non exhaustive list of different separation and identification techniques, and oldest paper referencing the use of the method

Enzyme linked immunoassay (ELIZA)	Artificial antibodies are reacted with the mixture, which then combine to the molecule of interest to cause a visible change. Mostly used for identification, can have numerous cross-reactivities.	Comparison of results for quantitative determination of morphine by radioimmunoassay, enzyme immunoassay, and spectrofluorometry	1975
fluorescence polarization immunoassays	Similar to above, but antibody is combined with a fluorescent molecule which is affected by the antigen-antibody reaction to cause a change in fluorescence intensity.	Determination of cocaine and benzoylecgonine by derivatization with iodomethane-D3 or PFPA/HFIP in human blood and urine using GC/MS (EI or PCI mode)	1993
Radioimmunoassay (RIA)	Similar to enzyme linked immunoassays, but uses radiolabelled molecules for better detection.	Comparison of results for quantitative determination of morphine by radioimmunoassay, enzyme immunoassay, and spectrofluorometry	1975
spectrofluorometry	The use of fluorescent properties of molecules to measure their concentration within a solution. Beams of light, commonly in the UV region, are shone through the sample, then the intensity and wavelength of fluorescence are measured to identify and quantify the substance.	Comparison of results for quantitative determination of morphine by radioimmunoassay, enzyme immunoassay, and spectrofluorometry	1975

Table 1.1 d: Non exhaustive list of different separation and identification techniques, and oldest paper referencing the use of the method

Liquid-liquid extraction	The use of solvent to separate the molecule of interest from its matrix. The fluid matrix is shaken with a known volume of solvent and left to settle to discard the matrix. This process is repeated multiple times to extract as much as possible of the molecule of interest. Time consuming sample purification method.	GC/MS quantitation of benzoylecgonine following liquid-liquid extraction of urine	1993
Solid phase extraction (SPE)	A solid (commonly sorbent) phase packed in a column, through which a fluid matrix is passed for the molecule of interest to bind to the sorbent by intermolecular forces. Solvents are used to remove impurities by dissolving the contaminants preferentially. Finally, a solvent which has similar properties to the molecule of interest is used to extract the molecule.	Solid-phase extraction of drugs from biological tissues--a review	1992
Solid phase microextraction (SPME)	Similar to SPE (solid phase extraction), but done using small columns, usually shaped similarly to syringes, and much smaller volumes.	Aqueous phase hexylchloroformate derivatization and solid phase microextraction: determination of benzoylecgonine in urine by gas chromatography-quadrupole ion trap mass spectrometry	1999

Table 1.1 e: Non exhaustive list of different separation and identification techniques, and oldest paper referencing the use of the method

Silylation	Derivatisation of the molecule of interest via a silicon containing derivatisation molecule. Done to improve detection.	Simultaneous determination of opiates, cocaine and major metabolites of cocaine in human hair by gas chromatography/mass spectrometry (GC/MS)	1995
Enzyme hydrolysis	The use of enzymes to obtain the molecule of interest from a metabolite, usually a glucuronide derivative.	Simultaneous analysis of codeine, morphine, and heroin after B-glucuronidase hydrolysis	

1.2.2: Forensic toxicology – Definition and historic development

Forensic toxicology is defined as the study of toxicology applied to the legal field (Wyman, 2012). More simply, it is toxicological analysis performed in order to obtain results which are intended to aid criminal investigations (Wyman, 2012). Thus, a forensic toxicologist is employed by the courts to; detect, identify and quantify drugs; both legal and illegal; from human samples and suspected drug samples; to help clarify the substances' implication in the investigated incident. This implies the expertise of a chemist or pharmacologist, as opposed to forensic medicine which requires the knowledge of a medical doctor, and is laboratory based (Wyman, 2012).

In forensic toxicology, the main samples are of biological origin, mostly fluids such as urine, blood, saliva, and stomach contents (Drummer, 2007). Other samples such as liver and vitreous humour are also analysed (Drummer, 2007). This introduced another problem in analytical procedures, since the apparatus used is very sensitive

and thus easily damaged by large solid particles. Detection is also altered by the various molecules present in the sample. Before they are tested, samples are processed or cleaned to prevent such occurrences (Drummer, 2007). Other types of samples are used in forensics, including hair, nails, stomach contents, vitreous humour and body tissues such as liver (Drummer, 2004). Of these, it is mostly hair and nails which are collected also from living people, but these keratinous structures often do not show the present state, however a medical history of the patient may be deduced due to the slow nature of drug deposition in integuments (Drummer, 2004). Stomach contents could be tested in live persons who have undergone some sort of gastric lavage, but still is more commonplace for autopsies (Drummer, 2007). Vitreous humour and other body tissues are viable only for autopsy, and only blood, urine and sometimes gastric contents are taken antemortem, usually as part of intoxication treatment in hospital (Jones, 2005). Usually it is biological fluids, namely urine, saliva, sweat, blood, meconium; hair, and breath which are collected from living suspects (Hadland and Levy, 2016).

Blood is considered as one of the best samples for drug analysis, as it correlates with level of intoxication, however it poses some disadvantages. In forensic toxicology, the samples are often taken from cadavers (Jones, 2005), thus blood is often partly coagulated and pooled to the lowest part of the body. Coagulated blood is difficult to extract via a syringe as it has solid particles (Jones, 2005). Postmortem redistribution may also alter the obtained results, which would not represent the picture prior to death (Jones, 2005).

Urine, being a fluid that contains no cells and compartmentalised to the bladder eliminates such problems (Jones, 2005). For this reason, it is the preferred sample in forensic analysis, but still has drawbacks. Mainly, the concentration of urine is impacted by the volume produced, and thus may not reflect the blood concentration precisely (Jones, 2005). Urine usually contains no protein; thus, no digestion step is required. It contains various solutes other than the molecule of interest, which can impact the results obtained. Nonetheless, the ease of extraction from cadavers; and the non-invasive nature of the sample derivation from living suspects, surpass the somewhat reduced precision of the back calculation obtained from urine (Jones, 2005).

Urine is also preferred to blood in most situations, since blood is a cellular matrix, thus requiring either separation of cells from plasma or digestion, both of which are time consuming and could cause some losses (Jones, 2005). Saliva and sweat can also be tested for drugs, with several swab and patch kits being available (Tamama, 2021), but these matrices tend to contain low concentrations of the molecules of interest (Tamama, 2021, Navazesh and Ahmadieh, 2020).

The first purification procedures introduced involved mainly liquid-liquid extraction or solvent extraction, which implements the use of a solvent within which the molecule of interest dissolves at a high rate (Martin and Synge, 1941). This solvent is vigorously shaken with the urine, left to settle than the fluid containing the analyte is collected for analysis; this is repeated multiple times to enhance extraction (Drabinska, Mlynarz

et al., 2020). This process is both time consuming and wasteful, thus solid phase extraction was developed (Plotka-Wasylka, Szczepanska et al., 2015).

Solid phase extraction (SPE) involves the use of a solid matrix to aid the extraction of the molecule of interest (D'Ovidio, Bonelli et al., 2022). This solid is present as tightly packed beads or other material within a column. Presently, solid phase micro extraction (SPME) columns consist of a column the shaped similarly to catheter syringes, with the packed material near the tip end. These have the capacity of only a few millilitres. Thus, it is only one or two millilitres of the original sample which are processed, as opposed to prior methods. The packing material is chosen of properties that increase affinity between the packing material and the molecule of interest. When the sample passes through the column the molecule of interest remains in the packing material due to adsorption (D'Ovidio, Bonelli et al., 2022), while the water from urine with other solutes percolates from the column.

Solvents are still implemented, as other molecules may have affinity to the column and thus need to be removed, but the quantities are only 1-2 mL, as opposed to solvent extraction which uses multiple 10-20 mL volumes to the same end. The process is also faster, with the solvents percolating in a few minutes as opposed to the lengthy times required for fluids to separate in solvent extraction. The extraction is more efficient, as some traces of the compound of interest will remain in the original matrix during solvent extraction, while the SPE column is more adherent to the desired molecule (D'Ovidio, Bonelli et al., 2022). The process is illustrated in Figure 1.1.

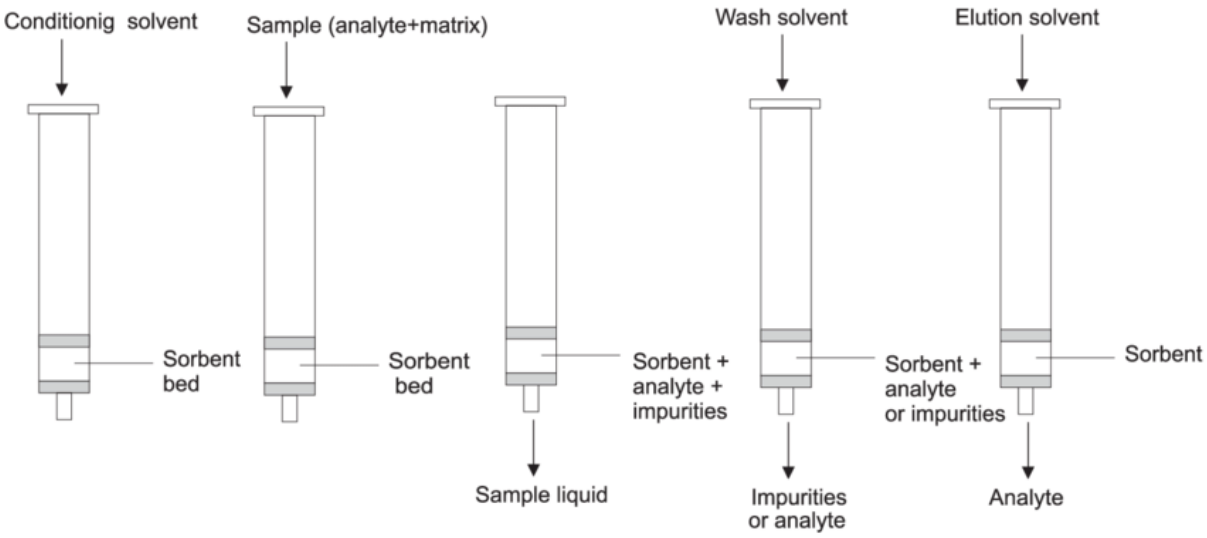


Figure 1.1: Overview of SPE column use (Zwir-Ferenc and Bizuik, 2006).

In this present study, the SPE column found in the kits used is manufactured by Agilent Technologies, of type Agilent Bond Elut Plexa PCX. This column is defined by the manufacturer as a strong cation exchanger with mixed sorbent mode characteristics (Agilent Technologies, 2011). This means that molecules of a net positive charge bind to the column resin, while eliminating other solutes. Thus, the resin is negatively charged and attracts basic molecules (Pereira Barbosa, Tchieta et al., 2013). The advantages of these columns over other commercially available SPE columns, as stated by the manufacturer (Agilent Technologies, 2011), are the more homogenous particle size having Gaussian distribution of the packing material which increase flow rate and prevent blockages, as compared to Water Oasis HLBn (Agilent Technologies, 2011). The Plexa PCX columns are marketed for the extraction and clean-up of non-polar and polar basic compounds (Agilent Technologies, 2011).

The physical and chemical characteristics of the drug are key to ensuring a successful drug analysis. At a pK_a of 8.6 cocaine is a weak base. Its main metabolites: ecgonine methyl ester is a strong base with pK_a 9.57 while benzoylecgonine has a strongly basic pK_a of 10.82 (Mella, Schweitzer et al., 2018). Benzoylecgonine has another pK_a at 3.35 (Mella, Schweitzer et al., 2018), which is acidic, thus may introduce some variable results. This predisposition is reduced with the use of an acidic buffer, which is available as part of the kit. The addition of acid favours the basic conformation of benzoylecgonine. Ecgonine methyle ester was not tested for as the kit used is not marketed for this metabolite. Benzoylecgonine is the main metabolite tested, while cocaethylene is a secondary metabolite which forms only in the presence of alcohol through transesterification in the liver. Molecular structures are shown in Figure 1.2.

Heroin is a strongly basic molecule, at pK_a 7.6 (Merves, Goldberger, 2005). On the other hand, morphine has a pK_a of 8.2 (Milella, D'Ottavio et al., 2023); and the main metabolite 6-monoacetyl morphine has 2 pK_a values, pK_a 8.0 most acidic and 9.4 most basic (Zoumpouli, Souza et al., 2020). These are all basic substances. These molecules are shown in Figure 1.1 as part of a hydrolysis pathway.

Thus, the Plexa PCX column is ideal to extract the molecules of interest from the biological matrix as they are all basic.

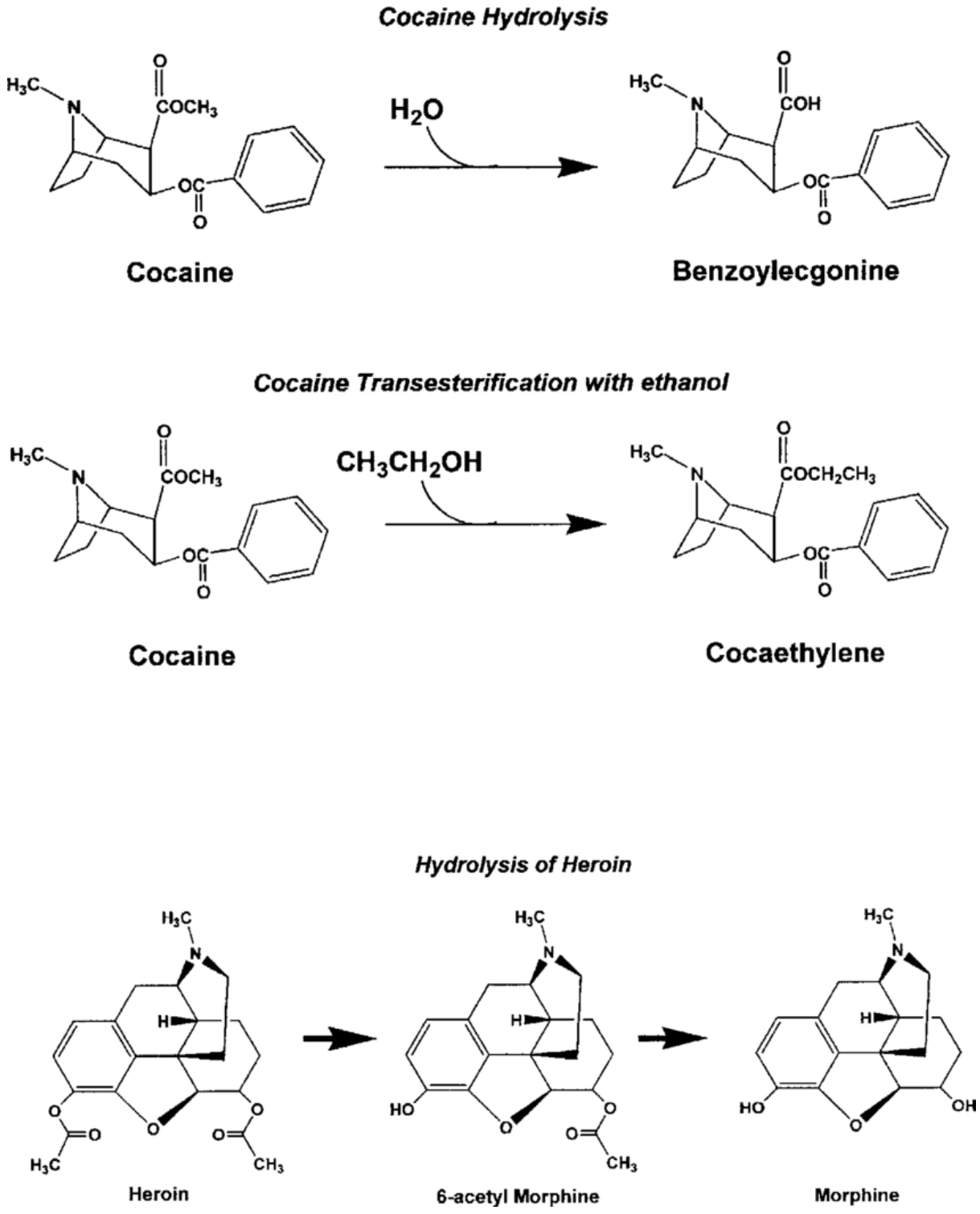


Figure 1.2: Cocaine, benzoylecgonine, cocaethylene, heroin, 6-monoacetylmorphine and morphine molecules with main metabolic conversions (Redinbo, Bencharit et al. 2003).

1.2.3: Challenges of drug detection and analysis

Drugs undergo metabolism from the time of absorption, so it is practically impossible to find the original quantity of the parent drug taken in a biological sample. This process also implies one can find one or more metabolites of the original compound, which may or may not have pharmacological effects. Regardless of such effects, the toxicologist must quantify both pure parent compound and the metabolites in order to accurately conclude the actual quantity present at the time of intoxication. The ratios of parent compound to metabolite can also indicate the time which has passed, and thus the approximate time of ingestion. This is the case for cocaine, which is metabolised to benzoylecgonine and to cocaethylene (in the presence of alcohol), all of which are present in the obtained samples. Cocaine has a half-life of 35 minutes to 1.5 h in the body (Preston, Epstein et al., 2002), benzoylecgonine 7.5 h (Preston, Epstein et al., 2002) and cocaethylene at 149 minutes (Hart, Jatlow, 2000). Thus, all compounds can be quantified in order to estimate back calculations.

Heroin poses greater challenges, since it is rapidly metabolised within minutes. Heroin is practically impossible to detect due to fast metabolism, thus confirmation of its use depends solely on metabolites. These are 6-monoacetyl morphine (6-MAM), which has a half-life of half an hour, and morphine (Milella, D'Ottavio et al., 2023). Morphine is non-specific, as many other opiates convert to this compound upon metabolism, so heroin detection is dependent on the narrow time frame, when 6-MAM is being eliminated in urine (Milella, D'Ottavio et al., 2023). Apart from this, opiates are generally eliminated as large glucuronide metabolites, which must be broken down

prior to analysis by GC/MS (Barroso, Gallardo et al., 2011). This breakdown can be performed enzymatically, as in the current study, or via acid, with enzymes however this is less efficient (Barroso, Gallardo et al., 2011).

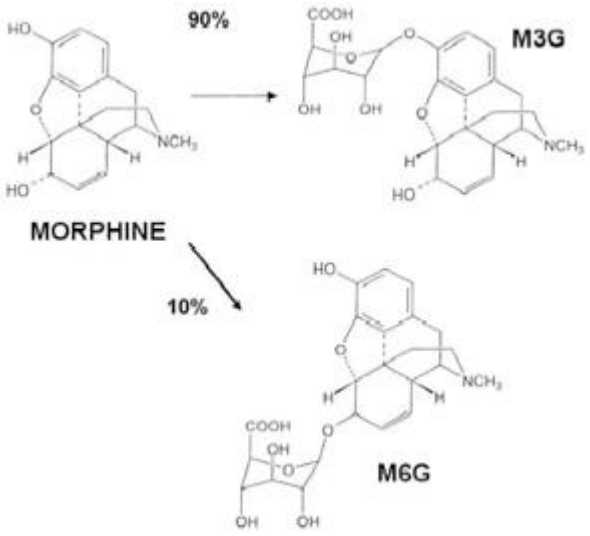


Figure 1.3 Glucuronide derivatives of morphine (De Gregori, De Gregori et al., 2012).

Another caveat to detection via GC/MS is the volatility and stability of the molecules to be detected. Organic molecules are often thermally unstable, and thus are predisposed to decomposition in the gas chromatography apparatus (Gumbi, Moodley et al., 2019). Derivatisation to silyl compounds is thus used to prevent such an occurrence, retaining the original molecular structure throughout the molecule's passage in the column (Gumbi, Moodley et al., 2019). The fragments produced from silylated compounds are also more stable, and thus enhance the detection limits of the GC/MS (Gumbi, Moodley et al., 2019). In this specific study compounds were silylated via SBSTFA (Selectra-Sil N,O-bis(trimethylsilyl) trifluoroacetamide molecule) included in

the kit. This agent is more reactive than other silylating agents and is rendered less reactive to hydroxyl groups via 1% TMCS, or trimethylchlorosilane (Lin, Wang et al., 2016). This ensures the reaction is complete, and that no side reactions occur (Lin, Wang et al., 2016).

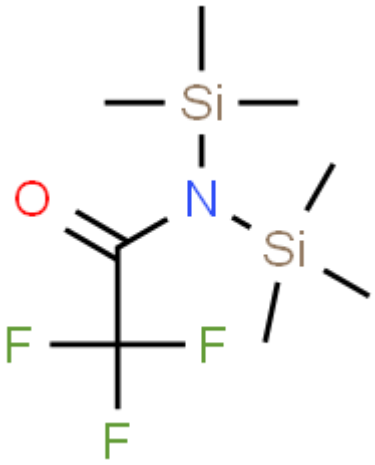


Figure 1.4: Selectra-Sil N,O-bis(trimethylsilyl)trifluoroacetamide molecule (Retrieved from: http://www.Chemspider.com on 15/11/2023)

Selectra-Sil BSTFA, or N,O-bis(trimethylsilyl)trifluoroacetamide, is a silylating agent used widely to derivatise compounds for GC/MS analysis (Lin, Wang et al., 2008). Derivatisation is required by compounds which have active hydrogen atoms in their molecular structure, found in polar carboxyl, amine and hydroxyl groups amongst others (Lin, Wang et al., 2008). Such groups make molecules within the compound more strongly attracted to one another, thus increasing boiling point (Lin, Wang et al., 2008). Hydrogen bonds and reactions with the components of the apparatus further hinder vaporisation and motility within the system, causing peak shrinkage or tailing (Lin, Wang et al., 2008). This is problematic for detection via GC-MS as to enter the GC

a compound must be volatilised, and very often complex organic molecules decompose at high temperatures.

SBSTFA (Selectra-Sil BSTFA N,O-bis(trimethylsilyl)trifluoroacetamide) is reacted with the compound of interest at the hydrogen bond, removing the active hydrogen atoms, making the substance more volatile (Lin, Wang et al., 2008). Silylation provides additional advantages over other derivatisation techniques, one being that it does not require further clean-up after the derivatisation step. The reaction is made easier as BSTFA has a strong stable leaving group, driving the reaction forward (Lin, Wang et al., 2008). BSTFA does not interfere with the GC, allowing the apparatus to produce clear results with distinct retention times (Lin, Wang et al., 2008). This is because the left-over compounds are much more volatile than other silylating agents, allowing easy dispersal of both remaining BSTFA and products of reaction (Lin, Wang et al., 2008).

All these variables introduce uncertainties. In order to reduce the impact of uncertainties and thus increase robustness of data, the validation of methodology is essential.

1.2.4: Validation in forensic toxicology

Validation is required by laboratories in order to confirm that the used methods are reaching the desired results (Doyle, 2019). This is of relevance in forensic toxicology since the concentrations encountered are usually very low, thus requiring much more effort for accurate results to be obtained (Doyle, 2019). This study entails partial validation, since the complete validation was carried out by the manufacturer prior to placing the product on the market (Eureka, 2022). Complete validation includes sensitivity and selectivity studies, mainly carried out to detect the lowest level of detection and to identify any cross reactivity, respectively (SWGTOX, 2013).

In this specific study, sensitivity and selectivity studies were omitted since they were confirmed by the manufacturer. For the current study, the limit of quantitation was considered as being the lowest concentration of the used calibrators which contains the molecule, namely calibrator level 2 as level 1 is a blank. This is at 50 ng/mL for all analytes. The manufacturer states the level of detection is as low as 5 ng/mL, indicating the method is robust (Eureka, 2022). For the purpose of this present study, the LoD will be equal to the LoQ, at 50 ng/mL. In partial validation, two very important parameters that were measured were the accuracy and precision (SWGTOX, 2013).

Accuracy is defined as the trueness of measurement, or how close to the actual value of measured value (SWGTOX, 2013). This was measured since it may vary from the inhouse apparatus of the manufacturer to the GC/MS used in the laboratory. Accuracy testing ensures that when applied inhouse, the method still functions up to the standards of the manufacturer (SWGTOX, 2013).

Precision is described as the closeness of several consecutive measurements of the same object are to one another (SWGTOX, 2013). In this case, the closeness of the detected concentrations of several samples of the same concentration (SWGTOX, 2013). This testing ensures there is no excessive variability in results (SWGTOX, 2013).

Both accuracy and precision are dependent on a few factors, including; the apparatus including its calibration and overall function; the operator carrying out the tests; the methodology; the condition of the reagents (SWGTOX, 2013). The method was followed as given by the manufacturer, as are the storage instructions and expiry dates. This leaves the apparatus and the operator as the main variables in the study, which influenced the results (SWGTOX, 2013).

Both parameters were measured intraday and inter-day, to observe if there are significant changes in measurement (SWGTOX 2013). This helped to detect any variability in measurement.

In this study, the criteria verified were: selectivity, linearity, working range, limit of detection, limit of quantitation, repeatability, precision, trueness (accuracy), robustness. For the purpose of the study LOD and LOQ were considered as the same, which is the lowest available calibrator concentration for each substance as stated in the appendices. It is worth noting that by manufacturer's information the LOD can be as low as 5ng/mL, as opposed to the 50ng/mL of the calibrator (Eureka, 2022).

Linearity was confirmed from the calibration curves Rf value, which was not accepted at lower than 0.99. Linearity ensured that the apparatus accurately calculates concentration from the signal. Working range was as indicated by the calibrators, with calibrator level 2 as the lowest concentration and calibrator level 5 as the highest concentration of the working range. The working range outlined the concentrations which could be confidently measured via the used method within desirable accuracy and precision parameters (SWGTOX, 2013).

Selectivity is defined as the ability to differentiate the analyte of interest from other compounds. This was achieved in the study by performing the preanalytical procedure for each molecule to detect the retention time and the obtained spectrum for the respective molecules. The manufacturer specified how the spectra should look and which peaks to use for quantitation, but the spectra obtained from the preanalytical were considered as more accurate as they were determined via the local apparatus (SWGTOX, 2013).

Precision was tested via the use of lyophilised solutions. The manufacturer produces these in two concentrations, both of which were tested for intraday and interday precision. Accuracy was calculated from the same tests, as the theoretical concentration of the lyophils was known (Eureka, 2022).

Sensitivity was observed from urine samples which were previously tested, to confirm the apparatus can detect the analytes of interest in a true matrix.

1.3.1 Literature review related to cocaine and heroin detection using GC-MS

The literature review was performed in 2 stages. First, MeSH terms Cocaine OR Heroin AND Urine OR Urinalysis AND GC-MS OR Toxicology were researched on the Academic Search Ultimate EBSCO, ProQuest Central and PubMed Central databases, with the searches resulting in a total of 26281 articles, of which 304 were deemed relevant to the study. The terms heroin and cocaine were searched on OAR@UM database, with a total of 11 texts being relevant.

In the second search, the terms "determination of cocaine in forensic analysis" and "determination of opiates in forensic analysis" were searched on PubMed, retrieving a total of 509 results. Of these, 206 were considered relevant. These are listed in Appendix 1.

Other terms searched on Google Scholar included; Solid Phase Extraction, Silylation, SBSTFA, Derivatisation for further understanding of the procedure.

The oldest retrieved title mentioned Gas Liquid Chromatography, a type of gas chromatography setup, was from 1974 (Cimbura and Kofoed). Gloger and Neumann (1983) experimented with packed columns, this type of column was not used often, and is not commonly used. Harris, Hamilton et al. (1979) mentions the use of mass spectrometry to determine the molecular weight of morphine and its derivatives, thus use of MS was gaining traction. The earliest text mentioning the use of GC/MS is by

Griesemer, Liu et al., published in 1983, detailed the detection of cocaine and benzoylecgonine in post mortem fluids. This showed the technique was well established and gaining traction beyond academia, entering routine use in diverse laboratories. Cone, Menchen et al. (1988) is the earliest text which mentioned the use of GC/MS as a comparison test to confirm the efficiency of another procedure. This indicated that by the end of the 1980s GC/MS was well established and considered with high regard. By the 90s, GC/MS was being used for large studies involving national drug use patterns (Schiwy-Bochat, Bogusz et al., 1995; Marquet, Delpla et al., 1998), workplace testing (Goldberger and Cone, 1994), clinical trials for immunoassays (Cone, Yuosefnejad et al., 1990; Cone, Dickerson et al., 1992; Cone, Dickerson et al., 1993; Crouch, Cheever et al., 1998).

Nowadays the technique is well established, and used in many areas. Forensic toxicology (Serafin, Paulemon et al., 2017) is one of many; where the drug concentration is obtained from a biological matrix, popularly urine or blood, often from the dead. This can help in determining cause of death, or if someone was intoxicated during an incident. Other matrices, including hair, nails, liver, muscle, brain and stomach contents may be used (Drummer, 2007), albeit the formerly mentioned are the most common choices. Urine testing is also performed in hospitals as part of autopsies to determine cause of death (Toshiko, Shiomi, et al., 2021). Clinical toxicology is another application (Sanchez-Gonzales, Garcia-Carballal et al., 2016), whereby the samples are from living patients under treatment for suspected use. Drug addiction treatment programs use urine samples to monitor drug use and treatment

efficiency (Gastberger, Baumgartner et al., 2019), statistical data related to drug use (Maurer, Vergalito et al., 2021), workplace drug testing (Carney, Wolf et al., 2012), sports doping (Magura, Lee-Easton et al., 2023), and pain medication monitoring (Carney, Wolf et al., 2012) amongst others. Urine drug testing is implemented in all scenarios where detecting and quantitating drug use can be of benefit to reaching the end goal. Urine is considered as one of the easiest matrices to process (Drummer, 2007), and is easy to obtain from both living and dead subjects. In the living it is seen as one of the least invasive, but can cause issues of privacy as the person has to be monitored during sample provision (Magura, Lee-Easton et al., 2023); but nonetheless preferred to blood, which is invasive and requires a complex procedure for collection (Feltraco Lizot, Cezimbra da Silva et al., 2019). Thus, the trust of the scientific community in GC/MS and the relative ease of urine collection have made urinalysis via GC/MS a popular drug screening method.

However, science is always evolving and changing, as are human preferences. In recent times, techniques such as LC/MS-MS (Lehmann, Keiliba et al., 2016; Sanchez-Gonzales, Garcia-Carballal et al., 2017; Yang, Liu et al., 2017; Johansen, and Bhatia, 2007; Picht, Beck et al. 2018; Feltraco Lizot, da Silva et al., 2019; Jakobsson, Truver et al., 2021), HPLC (Fernandez, Vázquez et al., 200 Fernandez; Regenjo et al., 2015), and LC-MS (Li, Shen et al., 2013) are gaining traction. These are claimed to be less time consuming and complex procedures, but are still as effective as GC-MS. GC-MS is still sought as the gold standard in research trials, with many studies using it as a comparator for the device being studied (Holler, Bosy et al., 2004; Moody, Fang et al.; 2006, Lu, Taylor et

al., 2006; Choodum and Nic Daeid, 2011; Sundstrom, Carney, Wolf et al., 2012; Pelander et al., 2015; Tanaka, Yoshizumi et al., 2021).

Samples require preparation prior to analysis, and in this study solid phase extraction was used to clean the sample from impurities. Moore, Brown et al. (1992) is the earliest retrieved study which used SPE, and this remained in use up to recent years (Lehmann, Kieliba et al., 2017; Sanchez-Gonzales, Bevalot, et al., 2014; Garcia Carballal et al., 2016; Yang, Liu, 2017; Li, Swortwood et al., 2021). SPE implements a column packed with sorbent material, to which the molecule of interest adsorbs; then solvents are pipetted to clean the sample, which is then dried and collected via a final solvent in which the molecule of interest preferentially dissolves. Other methods for sample purification include thin layer chromatography (De Carvalho, Tosado et al., 2016), Supercritical fluid extraction (Allen and Oliver, 2000), and liquid-liquid extraction (Gerlits, 1993).

The texts retrieved in the literature review using the key words listed above did not refer to the validation of a commercial kit. Most articles focussed on defining an inhouse devised methodology using various chemicals. These studies referred to specific chemical solutions and concentrations, as they were prepared within the facility (Argo, Zerbo et al., 2022; Chee-Yuen, Zafarina et al., 2019; Populeanu, Ionica et al., 2019; Agius, Nadulski, 2014; Smith, Nichols et al., 2014).

The inhouse methods were comparable to the kit methodology, since they also involved; sample buffering, clean-up via SPME columns which contain a packing material to which the analyte of interest adheres to, column conditioning prior to sample filtering through the column, and further clean-up via solvents followed by final extraction from the column using a solvent and drying with a gas flow (Knight, Puet et al., 2014; Rosado, Goncalves et al., 2017).

Some studies also used BSTFA similarly to the kits used for the study (here labelled as Reagent M in both kits used) (Yau, Dominowski et al., 2012; Lopez, Bermejo et al., 2009). The use of similar derivatisation increases comparability of methods, as the molecule used for this process dictates the stability levels of the molecule within the GC/MS apparatus; and, the fragmentation pattern, thus the spectrum. The kits fail to list the chemical identity of the main clean-up reagent solution; thus, method comparison is limited to the general procedure.

There may be several reasons for lack of discussion about such kits in the literature. Commercial kits are a recent introduction which may not be available to all premises; many facilities may not see a reason to validate a kit that was previously validated to be marketed; laboratories keep validation studies as classified data; method validation is not mandatory for all laboratories (Doyle, 2019), but no data is available on this subject.

Over the years various complex chromatographic techniques have been developed. With the advent of Gas Chromatography Mass Spectrometry (GC/MS), systems such as Fourier transform infrared spectroscopy (FTIR), high performance liquid chromatography (HPLC) (Larsen and Tebbett, 1992), nuclear magnetic resonance (NMR), x-ray diffraction, scanning electron microscopy (SEM), Raman Spectroscopy, UV-Vis (Siek, Osiewicz, et al., 1976), infrared spectroscopy (Beliaev, Soroki et al., 1997) , Electrospray ionization mass spectrometry (ESI-MS), Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), gas chromatography flame ionisation detection (GC-FID) (Fernandez, Aldonza et al., 2006) and Liquid chromatography-mass spectrometry (LC-MS) (Maurer, 1998) were also being developed.

Various methods can be applied to urinalysis, including ELISAimmunoassays, which are often scrutinised and thus more thoroughly validated by laboratories that implement their use (Agius, Nadulski, 2014). In such validation studies GC/MS is used as a confirmatory test, further proving the trust of the scientific community in GC/MS (Agius, Nadulski, 2014).

Eureka has produced the forensic diagnostic kits since the early 2000s, and is externally accredited, giving assurance to customers about its products (Eureka, 2022). This, however, fails to ascertain that the kit functions up to the standard desired by the laboratory. The performance of the method depends not only on the kit, but also on the available apparatus and the operator, and how these interact together in giving

the result (SWGTOX, 2013; Doyle, 2019). The company R&D laboratories may not the best comparison for the kit's performance as they would have invested in the latest equipment and highly trained personnel. The kits are externally accredited, increasing the reliability (Eureka, 2022). This does not eliminate the necessity of the need of on-site validation, since this will confirm how the kit works on site.

Of the mentioned techniques, Raman spectroscopy is the least complex, often found as a handheld device used on the original sample without any workup retaining the original sample (Mojica, Dai, 2022), it uses light refraction to detect bonds within molecules (Saletnik, Saletnic et al., 2021). However, this is not considered as a confirmatory test as it only gives an indication about structure and the lack of expertise makes results questionable (Kranenburg, Verduin et al., 2020). It is often used when large quantities of suspect drug material are found by initial responders on site (Mojica, Dai, 2022), then the substance is tested via more complex analysis in the laboratory. This is a rapid and cheap alternative to running blind chemical analysis, as then the laboratory would only need to confirm the Raman spectrum, reducing time and resources spent.

Another preliminary test employed by laboratories is the immunoassay. Immunoassays use artificial antibodies which bind to the drug of interest to produce a visual change to the matrix (Douglas, Gorley et al., 2009). Nowadays these are found as cassettes testing for various analytes simultaneously, using just a drop of sample (Moody, Fang et al. 2006). For biological samples no excessive workup is required, fluids often being

analysed in their original state, and results are obtained in a few minutes as opposed to spectroscopic and combination methods (Holler, Bosy et al., 2004). A great shortcoming is the high incidence of cross reactivity for many such cassettes, thus confirmatory tests being mandatory in the forensic sector when this method is used.

GC-MS requires a degree of sample processing, depending on the sample in question, with the process including: protein digestion (when present), removal of compounds which can interfere with readings or purification, removal of solvent or drying, derivatisation to improve detection by the apparatus and introduction and processing in the GC- MS (Drummer, 2007). This process requires a predefined procedure and the use of various chemicals (Drummer, 2007). This can be developed inhouse or acquired from other laboratories, but it is significantly time consuming.

As toxicology gained solid ground in numerous areas, including forensics, industry saw a new area for innovation. In the early 2000s, kits for the analysis of biological fluids started emerging on the market (Eureka, 2023; Thermofisher, 2023). These eliminated the need to develop methods from scratch and to source the required solvents and reagents separately, as the kit contained all chemical reagents needed and was marketed including the methodology. In this study Eureka Division test kits will be employed.

Other companies such as Biobase produce commercial kits for urinalysis via GC/MS, which are also certified, and Agilent with test kits for LC/MS. Other companies, including Thermofisher and Agilent produce SPE columns. Commercially produced

columns ensure the same raw materials are being used, thus extraction is uniform from one test to another. These also reduce human errors of measurement off the matrix volume. The columns by Agilent are included in the kits.

Ready to use kits, similarly to in house methods, use a series of solutions to extract the analyte of interest and remove any substance that may interfere with the detection of said analyte (Eureka, 2023; Thermofisher, 2023). The major difference is that the solutions are produced in masse by the manufacturer, eliminating the often-tedious weighing and measuring procedures that may result in additional errors when preparing stock solutions. The solid phase microextraction columns are chosen by the manufacturer to optimise extraction efficiency, further reducing variability (Eureka, 2023). The main apparatus to be monitored are the pipettes (which were calibrated) and the GC/MS apparatus chosen by the laboratory, as they are a source of error (SWGTOX, 2013).

The methodology provided by the manufacturer provided all setting information, to ensure the kit is used in optimum conditions (Eureka, 2023). Good settings are essential to allow complete separation of ions and allow accurate detection.

1.3.2 Gaps in literature

As already stated, from the studies retrieved, none mentioned using a commercial kit. This clashes with the supposition that a ready-made kit with easy-to-follow instructions requiring only the analytical equipment and basic laboratory glassware as adjuncts for use should be in high demand by analytical laboratories. One theory is that these kits are not scrutinized by their buyers, and are immediately used for the indicated purpose without any validation process. Another theory would be that such validation studies remain unpublished as they are considered in-house documentation, and thus classified information not to be publicised. Various reasons may be presented for not validating a commercial kit, including; validation studies are carried out at multiple sites for R&D by the manufacturer prior to marketing; kit being CE licences thus verified to be fit for use by authorities (Eureka, 2019); manufacturer being generally trusted, and; no legal or formal obligation to do so. This, however, does not eliminate the fact that the kit may, in fact, function differently when used with the equipment available at another laboratory. In R&D of large companies, laboratories are generally equipped with the latest technology and highly maintained, which may not be the case in all premises. Apparatus such as GC/MS may vary in its function according to use. Thus, in-house testing can shed light on the actual functionality of the method onsite (SWGTOX, 2013).

Locally only two studies were published, one validation study by Bio-DNA and another by the University of Malta Forensic Laboratory, however these used an in-house

developed methodology (Cini, 2019; Sammut, 2004). Table 1.2 is a list of studies published by locals related to drug use and testing.

Table 1.2 a: List of locally published studies related to drug use and testing

Title	Author	Date	Description
Anomalous urine drug testing results in Maltese heroin addicts.	James Agius	04/09/2013	Thesis testing the efficiency of immunoassays to detect heroin use in urine samples. GC/MS and HPLC used as comparison.
Synthetic Drugs of Abuse Exhibiting Kinetic and Non-Kinetic Toxicology	Sarah Xuereb, Janis Vella Szijj, Lilian M. Azzopardi	2019	Literature review on the effects of cocaine, MDMA and synthetic cannabinoids.
Validation of Methods for Testing Drugs of Abuse	Michaela Cini	2019	Validation of inhouse GC/MS method.
An Overview of Suspected and Acute Poisoning in Mater Dei Hospital	Robert Camilleri	03/2014	Statistical study of acute drug poisoning patients, listing symptoms and correlating symptoms with drugs used by the patients.
Guidelines for European workplace drug and alcohol testing in hair	Ronald Agius and Pascal Kintz	05/11/2010	Validation of a workplace drug testing procedure using hair.
Significantly increased detection rate of drugs of abuse in urine following the introduction of new German driving licence re-granting guidelines	Agius, Ronald Nadulski, Thomas Kahl, Hans-Gerhard Dufaux, Bertin .	16/11/2011	Validation of am immunoassay drug testing procedure compared to GC/MS.

Table 1.2 b: List of locally published studies related to drug use and testing

Validation of LUCIO®-Direct-ELIZA kits for the detection of drugs of abuse in urine: Application to the new German driving licence re-granting guidelines	Ronald Agius, Thomas Nadulski, Christine Moore	08/11/2011	Validation of new immunoassay drug detection method against GC/MS.
Medicines and Drugs on the Job	Mary Anne Ciappara	04/1988	Discussing legalisation of workplace drug testing.
Perceptions of Students on Alcohol and Drugs	Nicole Bugeja	05/2020	Cohort study related to perceptions on drug use amongst university students.
Risk factors for adolescents developing substance use disorders; what should our prevention programs be targeting?	Nigel Camilleri, Andrea Saliba	2018	Article discussing drug use initiation amongst minors and risk factors. Statistical data on drug use.
Drug Offences: A Legal Analysis of Procedural Aspects and Punishment	Mariella Said	06/2006	Thesis on legal implications of drug use and possession.
Recreational drug use and the emerging challenges of psychoactive substances in Malta – A case series	Jeffrey Bonnici, James Coulson, Dorothy Gauci	2017	Study on admissions to Mater Dei emergency ward related to drug use.
The procedures and remedies related to drug use and abuse: a comparative analysis	Denise Apap	04/2017	Thesis on the legal aspects of drug use, and legal interventions to drug abuse.
Drug Rehabilitation: Intersectionality between Law, Medicine and the Self	Darlene May Gauci	04/2020	Qualitative study on drug addiction and rehabilitation.
Pregnancy in maltese drug-abusers: a socio-biological study	C. Savona-Ventura	30/01/2004	Statistical study of pregnancy and drug use.
A Comparative study of the Forensic analysis of Heroin by different Capillary Electrophoretic Techniques	Godwin Sammut	09/2004	Validation study of an in-house procedure using GC/MS.

There is a lack of research in the forensic field (Doyle, 2019). This is unexpected as many countries require accreditation in the field (Doyle, 2019). Many premises may forgo accreditation due to the time and rigour involved, and the realisation that some changes required are beyond the laboratory personnel's control (Doyle, 2019). Validation is considered a very important procedure, which can buffer any objections and suspicions from outside sources even in the absence of full accreditation (Doyle, 2019). This is because validation proves that the used method is giving the expected results within an accepted range (SWGTOX, 2013).

1.4.1 Objectives of the Study

Thus, the aims of this present study were to design a validated procedure to be used by the University of Malta Forensic Analysis and Toxicology Laboratory when analysing urine samples for the presence of cocaine or opiates.

The main objective was to partially validate the commercially produced Eureka® Lab Division urine test kits for use within the laboratory in combination with available GC-MS equipment. Another objective was to confirm the method validated by estimating the urine concentration of cocaine or opiates present in samples obtained from cadavers.

1.5 Outline of the following chapters

The Methodology Chapter will first list the materials and reagents used, followed by the overview of how the kits were used in conjunction with the calibrators, controls and samples. Next, the actual analytical procedures for the kits are described, followed by the statistical equations used.

The Results Chapter provides the results obtained and the data processing and statistical analysis carried out on the raw data, including calibration curves and retention times.

The Discussion Chapter interprets the obtained results in detail and compares these to other published studies, followed by the Conclusion on the obtained results and suggestions for improvement.

Chapter 2

Methodology

2.1: Methodology

The aims of this study were to calculate the accuracy and precision of the results obtained when analysing urine samples with the kits manufactured by Eureka® Division on the GC/MS apparatus within the Toxicology Laboratory at University of Malta. The methodology described in this Chapter was provided by the manufacturer with the kits and was used to prepare the samples for analysis. No significant alterations were made to the procedure as given, thus ensuring consistency with the manufacturer's indications.

Figure 2.1 shows an overview of the validation plan, which summarises the testing plan followed. The respective kits, calibrators and controls are also listed in the chart for reference.

Figure 2.1a: Validation Plan Overview for cocaine urinalysis

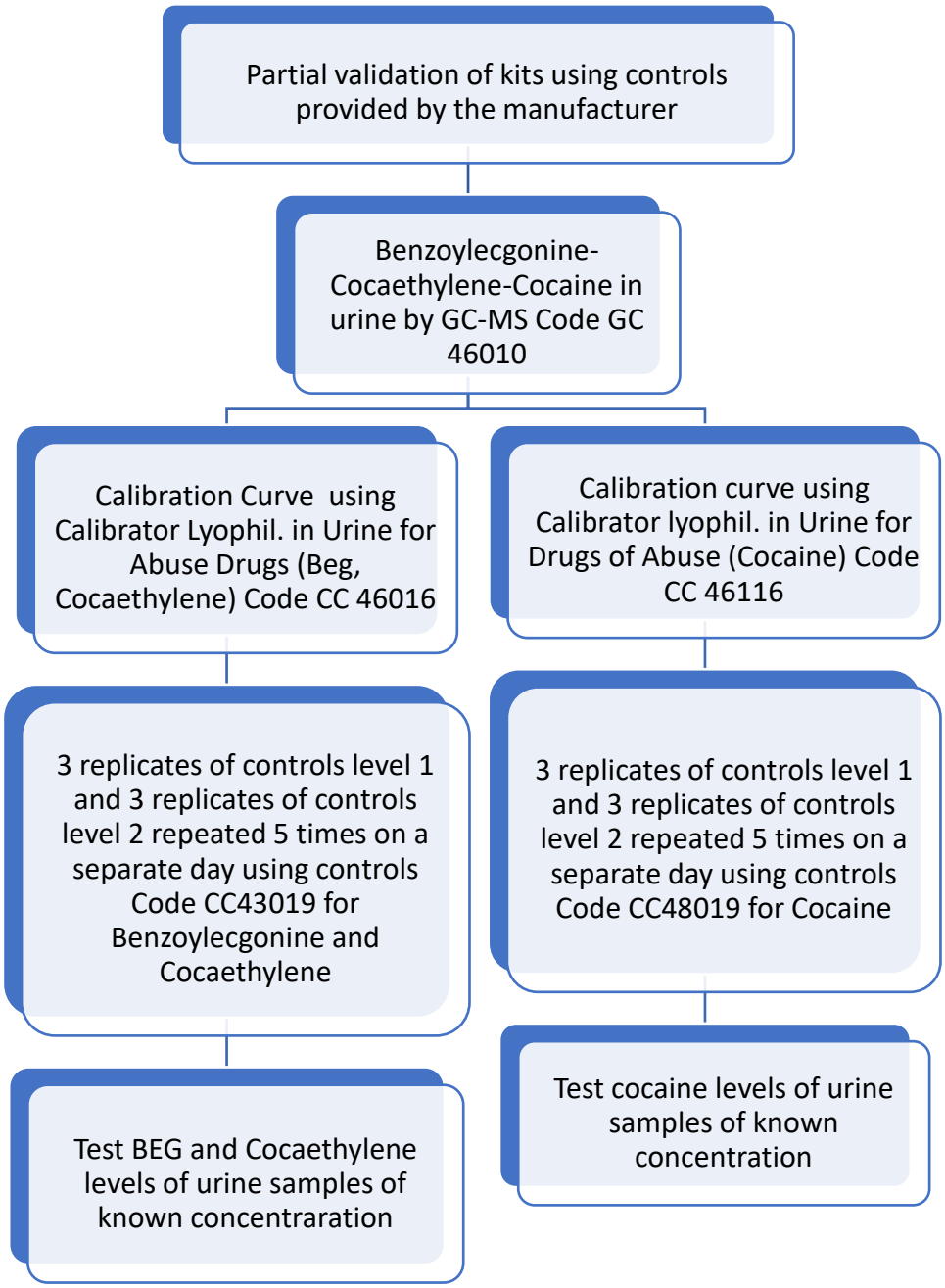
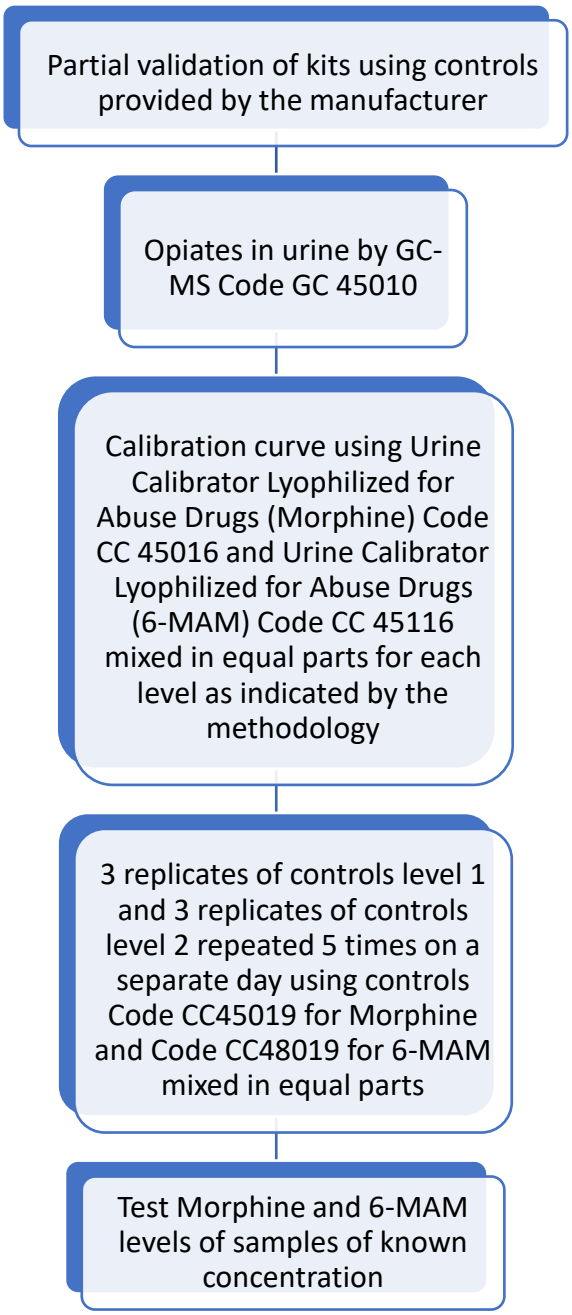


Figure 2.1b: Validation Plan Overview for opiate urinalysis



The urine samples used for the validation of this method were obtained from forensic cases of overdose which had been closed by the Courts of Malta. The permission to use these samples was obtained from the Inquiring Magistrate assigned the cases, Mater Dei Legal Office and CEO. The University of Malta Faculty of Medicine and

Surgery Ethics Committee (FREC) subsequently gave Ethics approval for this part of the study on 29th August 2023.

2.1.1 Materials and reagents

For this study the following materials and reagents were used, all of which were sourced from Eureka® Lab Division:

- Urine Calibrator Lyophilized for Abuse Drugs (Morphine, Dihydrocodeine, Codeine, Ethylmorphine) Code CC45016
- Urine Calibrator Lyophilized for Abuse Drugs (6-MAM) Code CC45116
- Urine Calibrator Lyophilized for Abuse Drugs (Cocaine) Code CC46116
- Urine Calibrator Lyophilized for Abuse Drugs (BEG, Cocaethylene) Code CC46016
- Control Lyophil. In Urine for Drugs of Abuse – Levels 1 and 2 (Morphine, Dihydrocodeine, Codeine, Ethylmorphine) Code CC45019
- Control Lyophil. In Urine Multiparametric for Abuse Drugs – Levels 1 and 2 (3,4-MDMA, 3,4-MDA, 3,4-MDE, Amphetamine, Methamphetamine, Ketamine, MBDB, Ephedrine, Pseudoephedrine, Norpseudoephedrine, BEG, Cocaethylene, Methadone) Code CC43019
- Control Lyophil. In Urine for Abuse of Drugs – Levels 1 and 2 (EDDP, 6-MAM, Cocaine) Code CC48019
- Benzoylecgonine (Cocaine's metabolite) – Cocaethylene - Cocaine in urine by GC-MS - Code GC46010 containing:
    - Reagent A – Conditioning Solution 1 and Wash Solution 2
    - Reagent B – Conditioning Solution 2

- Reagent C – Buffer Solution
- Reagent D – Wash Solution 1
- Reagent E – Eluting Solution
- Reagent F – Diluting Solution
- Reagent H – Test Solution Benzoylecgonine
- Reagent I – Test Solution Cocaethylene
- Reagent K – Test Solution Cocaine
- Reagent J – Internal Standard Solution
- Reagent M
- Clean-up Columns Phase Plexa PCX 3CC 30mg provided by Agilent Technologies (part of the kit)

And,

- OPIATES in urine by GC-MS – Code GC45010 (Morphine, Dihydrocodeine, Codeine, Ethylmorphine, 6-Monoacethylmorphine) containing:
  - Reagent A – Hydrolysis Enzyme
  - Reagent B – Buffer Solution for hydrolysis
  - Reagent C – Conditioning Solution N° 1 and Wash Solution N° 3
  - Reagent D – Conditioning Solution N° 2
  - Reagent E – Buffer Solution, 1
  - Reagent F – Wash Solution N° 1
  - Reagent G – Wash Solution N° 2
  - Reagent H – Eluting Solution
  - Reagent I – Diluting Solution

- Reagent N – Test Solution
- Reagent S – Internal Standard Solution
- Reagent M
- Clean-up Columns Phase Plexa PCX 3CC 30mg provided by Agilent Technologies (part of the kit)

- GC/MS Hewlett Packard HP 6890 Series GC System combined with Hewlett Packard 5973 Mass Selective Detector
- Macherey-Nagel™ CHROMABOND™ Complete Vacuum Manifold
- VLM EVA-EC1-S Nitrogen Evaporator for drying samples
- Stuart Scientific Auto vortex SA6 Vortex mixer
- Eppendorf Reference 2-20 µL micropipette
- Eppendorf Reference 20-200 µL micropipette
- Pipetman Gilson EJ91027 100-1000 µL pipette
- Thermo Scientific Finnpipette® F2 1-5 mL pipette
- 50 µL Hamilton® Syringe
- Fisher Scientific Capped borosilicate Pyrex™ test tubes

2.1.2 Procedure Plan Overview for the determination of cocaine and its metabolites benzoylecgonine (BEG) and cocaethylene

In order to determine the concentration of cocaine and its metabolites benzoylecgonine (BEG) and cocaethylene, the methodology as described in section 2.2.2 was used. Kit Code GC46010 was used and calibrators Code CC46116 were used to derive the calibration curve for cocaine, while calibrators Code CC46016 were used to derive the calibration curves of BEG and cocaethylene (see Figure 2.1a).

Methodology 2.2.2 was then also used to carry out the accuracy and precision studies using Controls levels 1 and 2 Code CC48019 for cocaine and controls levels 1 and 2 Code CC43019 for BEG and cocaethylene. In subsequent steps the procedure described in section 2.2.2 was repeated on urine samples of known concentration in order to verify the accuracy and precision on actual samples (see Figure 2.1a).

Accuracy and precision were then tested using the same raw data, as for each the minimum quantity of repeats required is having three replicates in a single run and 5 separate runs, amounting to a total of 15 concentration values for each of Code CC48019 for cocaine and Code CC43019 for BEG and cocaethylene. The controls for cocaine Code CC48019 must be tested separately, resulting in fifteen level 1 and fifteen level 2 tests for the compound, while BEG and cocaethylene are in the same controls level 1 and 2 Code CC43019, with 15 samples of each concentration. This is explained in Table 2.1 in the appendices.

2.1.3 Procedure Plan Overview for the determination of morphine and 6-MAM

Controls Level 1 and 2 Code CC45019 and Code CC48019 for morphine and 6-MAM respectively, were used to carry out partial validation of Kit Code GC45010 used in section 2.2.1. The methodology for morphine and 6-MAM required the simultaneous use of controls for morphine and 6-MAM, which resulted in fifteen level 1 tests and fifteen level 2 tests. For the calibration curve of Kit Code GC45010 Calibrators Code CC45016 for morphine and calibrators Code CC45116 for 6-MAM were used in conjunction as described in method 2.2.1. This is summarised in Figure 2.1b. for testing, 1 mL of urine was used to test the levels of morphine and 6-MAM in the urine samples.

The raw data obtained was used to calculate both accuracy and precision, each required five repeats on separate days of three replicates of each concentration, amounting to fifteen total values for Control Level 1 and Control Level 2. As the methodology allowed mixing 500 µL of control for morphine and 500 µL control for 6-MAM only 15 total tests were carried out.

2.2.1 Methodology for testing morphine and 6-MAM

Procedure Plan Overview for the determination of morphine and its metabolite 6-MAM

In order to determine the levels of morphine and its metabolite 6-MAM, the following procedures were carried out.

Calibrators Code CC45016 and Calibrators Code CC45116 were used in order to prepare for the subsequent parts of the methodology as described in section 2.2.1 (i.e., the calibration curve to determine the concentration of morphine and 6-MAM). The methodology as described in section 2.2.1 was also used with Controls levels 1 and 2 Code CC45019 and Code CC48019 to determine accuracy and precision of the method.

Calibrator and Control Reconstitution

As per Manufacturer instructions, each single vial of either calibrator or control was left to set to room temperature (they need to be stored in the refrigerator between 2°C and 8°C when dry), then reconstituted using 5 mL of HPLC grade water using a 5 mL pipette. The mixtures were recapped with their rubber stopper, inverted and left to set for 5-10 min to allow complete dissolution. These were then used as per the procedures.

The analytical procedure was first performed on the calibrator solutions to derive a calibration curve. Appendix 2 contains the concentrations of the calibrators used for

the procedure. Next, the controls were tested in triplicates, five runs on separate days for CC45019 and CC48019, followed by a run with true urine samples.

Preanalytical Procedure

For the preanalytical procedure 450 µL of Reagent I and 50 µL Reagent N were pipetted with a 1 mL micropipette into a capped test tube. The mixture was vortexed for 20 s and dried under a Nitrogen flow. Then, 50 µL of Reagent M were measured with a Hamilton syringe and mixed with the dried mixture in the test tube. This was vortexed for 20 s and incubated at 70 °C for 20 min. The mixture was left to cool to room temperature, pipetted into a GC/MS vial with insert, and 1 µL of this solution was run on the GC/MS and the retention times of opiates were identified. The results were compared to the figure provided by the manufacturer to confirm they were similar. The procedure was continued after similarity was verified.

Analytical procedure

1000 µL Reagent A were mixed with 9000 µL of reagent B by inversion. Reagent B was measured with a 5 mL pipette, and mixed directly into the vial containing 1000 µL of Reagent A. Reagent S was diluted in a 1:10 ratio according to the quantity of samples to be tested, Reagent S was measured with a 20 µL micropipette, and diluted with HPLC grade water pipetted with a 200 µL micropipette. Calibrators, control lyophils and samples were prepared as indicated in Table 2.2 in the annexes. the calibration curve was derived on 5 points, using calibrators for opiates Code CC45116 and

calibrators for 6-MAM Code CC45016. The controls were prepared in triplicates on different days, and 1 mL of sample was used for urine sample testing. The lyophilised solutions were pipetted in capped test tubes with a 1000 µL micropipette set on 500 µL. 200 µL of reconstituted Reagent A was pipetted into each test tube with a 200 µL micropipette, and 20 µL of reconstituted Reagent S was pipetted into each test tube with a 20 µL micropipette. The mixtures were tested with universal indicator paper to ensure the pH was 5. The test tubes were capped, vortexed for 20 s and incubated at 50 °C for 3 h.

The samples were left to cool to room temperature, and 2000 µL Reagent E was pipetted into each tube. The mixtures were vortexed for 20 s. 1500 µL Reagent C were pipetted with a 5 mL pipette into the SPE columns, and after this fully percolated 2000 µL of Reagent D were pipetted in each column with a 5mL pipette and were left to percolate completely. The previously prepared calibrators/controls/samples were poured each into a separate column, and left to elute completely. To clean the samples from any impurities 2000 µL of Reagent F were pipetted into each column with a 5mL micropipette and left to elute completely, followed by 2000 µL of Reagent G pipetted into every column with a 5mL pipette, and after complete elution 2000 µL of Reagent C was pipetted into all columns with a 5mL pipette. Reagent C was eluted completely, and a vacuum of 5 inches Hg pressure was applied to the chamber on which the columns were fixed to remove all fluids from SPE columns. Clean test tubes were placed below the SPE columns and 1000 µL of Reagent H were pipetted in each column with a 5 mL pipette. The eluate was collected into the test tubes, with vacuum

pressure applied to collect all the sample. The samples were vortexed for 20 s and dried under a nitrogen flow. Then, 50 µL of Reagent M were measured with a Hamilton Syringe and pipetted into each test tube, after which the samples were vortexed for 20 s each and incubated at 70 °C for 20 min. The samples were cooled to room temperature, pipetted into separate GC/MS vial (with inserts) and Run on the GC/MS. The results were compared to the preanalytical test and the calibration curve obtained prior to the control runs. The spectra were provided by the manufacturer for comparison, and available on the database.

Gas Chromatography – Mass Spectrometer Settings

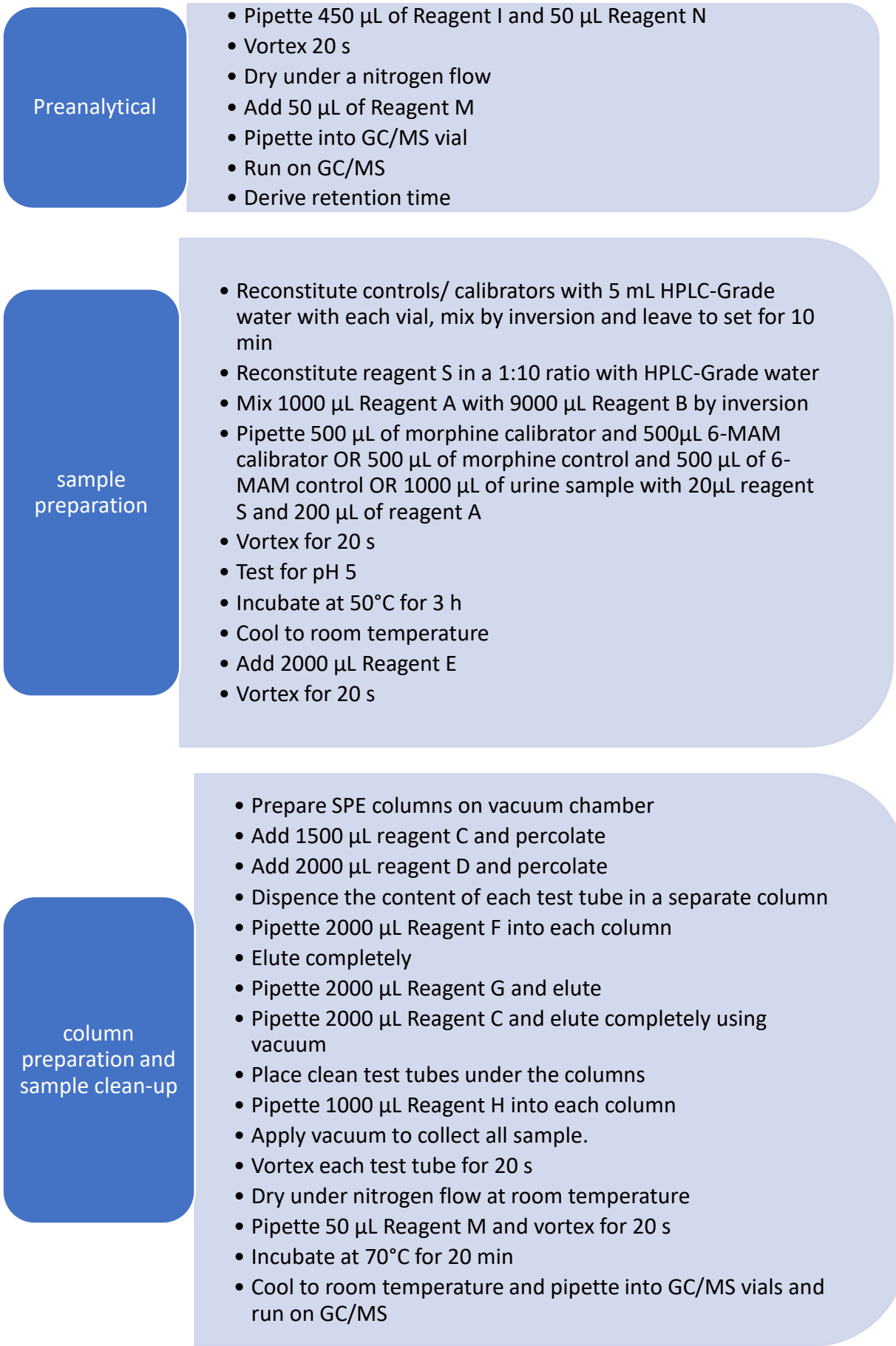
As per the manufacturer instructions the following settings were used for the runs: The Mass detector was set at a range between 78 and 400 m/z, temperature of mass 180 °C, Transfer line Temperature of 270 °C, Temperature manifold at 80 °C and the filament was turned on at 5 min for opiates.

The Gas Chromatograph was set as follows: VF-5ms Column 30 m x 0.25 mm, 0.25 microm was conditioned following manufacturer prescription; Injector's Temperature was set at 280 °C; Split Report Start: ON split 20, 0 min: OFF, 1 min: ON split 100, 3 min: ON split 20; Temperature Programmed: 120 °C x 3,25 min, 120-250°C at 20 °C/min, 250°C x 1 min, 250-300°C at 40 °C/min, 300 °C x 3 min (run 15 min); helium was pumped at 1 mL/min.

The internal standard had a target ion of 455 and qualifier ions of 440 and 414. A target ion of mass 429 and qualifier ions of 414 and 234 were used for morphine. For 6 monoacetyl morphine a target ion of 399 and qualifier ions 340 and 281 were used.

The retention times were 12.819 min for morphine, 13.144 min for 6 monoacetyl morphine and 13.362 min for the internal standard.

Figure 2.1 Flow Chart of Methodology for kit Code GC45010



2.2.2 Methodology for determination of cocaine, BEG and cocaethylene

Procedure Plan Overview for the determination of cocaine, BEG and cocaethylene

In order to determine the levels of cocaine and its metabolites BEG and cocaethylene, the following procedures need to be carried out.

Calibrators Code CC46116 and Calibrators Code CC46016 are used in order to prepare for the subsequent parts of the methodology as described in section 2.2.1 (i.e., the calibration curve to determine the concentration of cocaine, BEG and cocaethylene). The methodology as described in section 2.2.1 was also used with Controls levels 1 and 2 Code CC43019 (BEG and cocaethylene) and Code CC48019 (cocaine) in order to determine accuracy and precision of the method.

Preanalytical Procedure

For the preanalytical procedure 350 µL of Reagent F, 50 µL of reagent H, 50 µL of Reagent I and 50 µL of Reagent K were pipetted into a test tube with a 1000 µL pipette. The mixture was vortexed for 20 s and dried under a Nitrogen flow at room temperature. 50 µL reagent M were measured with a Hamilton Syringe and pipetted into the test tube, vortexed for 20 s and incubated at 70 °C for 20 min. The resulting mixture was left to cool to room temperature and pipetted into a GC/MS vial with insert with a 200 µL pipette. This was run on the GC/MS to obtain the retention times of cocaine, cocaethylene and benzoylecgonine on the available GC/MS apparatus. The

obtained results were compared to the spectra provided by the manufacturer to confirm the desired compounds were detected.

Analytical Procedure

For the analytical procedure Reagent J was reconstituted with HPLC grade water in a 1:100 ratio with a 20 µL micropipette to measure the required amount of Reagent J and a 200 µL micropipette to measure the water needed to make up the mixture. This was vortexed to mix. To prepare the test solutions, 1000 µL of calibrator/control/sample was measured using a 1000 µL micropipette and pipetted into separate capped test tubes. To each, 20 µL of reconstituted reagent J measured with a 20 µL pipette was added, followed by 2000 µL of reagent C pipetted using a 5 mL pipette as described in Table 2.3 in the annexes. Each mixture was vortexed for 20 s and tested with universal indicator paper to ensure the pH was 6.

The test tubes were vortexed for 20 s to mix and tested to ensure pH was approximately 6 with universal indicator paper.

To precondition the cleaning columns 1500 µL of Reagent A were pipetted with a 5 mL pipette and percolated drop by drop, followed by 2000 µL of Reagent B pipetted with a 5 mL pipette and percolated drop by drop. Once the columns were dry each sample was poured into in a separate preconditioned column and percolated.

For the clean-up 2000 µL of HPLC grade water were pipetted with a 5 mL pipette into each sample and percolated. After, 2000 µL of Reagent D were pipetted with a 5 mL pipette in each column and percolated, and 2000 µL of Reagent A were pipetted with a 5 mL pipette and percolated. The columns were dried with a vacuum system set at a pressure not higher than 5 mmHg.

The samples were collected in clean test tubes placed under the columns set-up. 1000 µL Reagent E were pipetted with a 5 mL pipette in each column and percolated, vacuum was used to collect all the samples completely, at a pressure no higher than 5 mmHg. The samples were vortexed for 20 s and dried under a Nitrogen Flow. After, 50 µL of Reagent M were measured with a Hamilton Syringe and pipetted into each test tube. The resulting mixtures were vortexed for 20 s and incubated for 20 min at 70°C. After they were cooled to room temperature and pipetted using a 200 µL micropipette into separate GC/MS vials with inserts and run on the GC/MS with the recommended settings. The results were compared to the calibration curve obtained from this procedure and the preanalytical, with the aid of the data provided by the manufacturer.

Gas Chromatography – Mass Spectrometer Settings

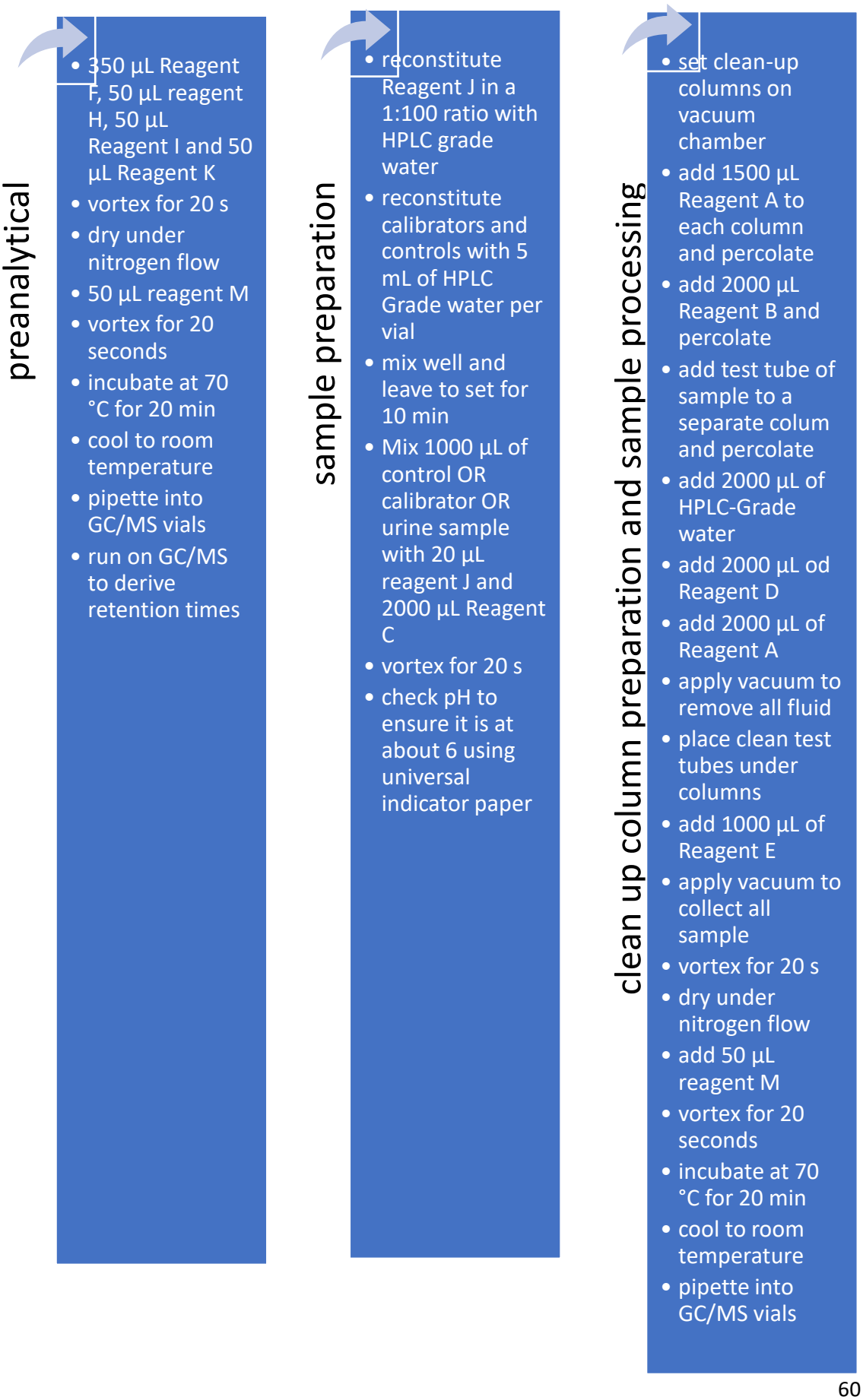
As per the manufacturer instructions the following settings were used for the runs: The Mass detector was set at a range between 78 and 400 m/z, temperature of mass 180 °C, Transfer line Temperature of 270 °C, Temperature manifold at 80 °C and the filament was turned on at 3 min for cocaine, cocaethylene and benzoylecgonine.

The gas chromatograph was set as follows: VF-5ms Column 30 m x 0,25 mm, 0,25 microm was conditioned following manufacturer prescription; Injector's Temperature was set at 280 °C; Split Report Start: ON split 20, 0 min: OFF, 1 min: ON split 100, 3 min: ON split 20; Temperature Programmed:120 °C x 3,25 min, 120-250 °C at 20 °C/min, 250°C x 1 min, 250-300 °C at 40 °C/min, 300 °C x 3 min (run 15 min); helium was pumped at 1 mL/min.

The target ion for atropine, used as an internal standard, is 124, with 94 and 361 used as qualifiers. For benzoylecgonine the target ion used was 240, and the qualifier ions were set at 256 and 361. The settings for cocaethylene were a target ion of 196 with qualifier ions of mass 212 and 317. For cocaine a target ion of 182 was used with qualifier ions of 82 and 303.

The retention times were 11.865 min for benzoylecgonine, 11.849 min for cocaethylene,11.578 min for cocaine and 11.697 min for atropine.

Figure 2.2 Methodology for Kit GC46010



2.2.3: Calibrator and Control Reconstitution

As per Manufacturer instructions, each single vial of either calibrator or control was left to set to room temperature (they need to be stored in the refrigerator between 2 °C and 8 °C when dry), then reconstituted using 5 mL of HPLC grade water using a 5 mL pipette. The mixtures were recapped with their rubber stopper, inverted and left to set for 5-10 min to allow complete dissolution. These were then used as per the procedures.

The analytical procedure was first performed on the calibrator solutions to derive a calibration curve. Appendix 2 contains the concentrations of the calibrators used for the procedure. Next, the controls were tested in triplicates, five runs on separate days for CC43019 and CC48019, followed by a run with true urine samples. The lyophilised solutions are certified reference material. For this study they were used for the accuracy and precision study, but they are given as part of the kit primarily to be used as a comparison when testing samples. Thus, no other reference material was used based on the assumption these lyophilised solutions should be of the desired accuracy levels.
For precision, a guideline by SWGTOX (2013) was used, and the minimum quantity of repeats required for partial validation was deemed sufficient for the study, as the kit was already tested by the manufacturer.

2.3 Data processing

Each sample was run on the GC/MS twice, in order to reduce inaccuracies introduced by the apparatus. The average of the two readings was calculated. This average was used for all subsequent calculations.

Three repeats were prepared for each run. For each molecule, five runs were performed for each molecule, each run on a separate day. Thus, data was processed in triplicates for intraday results and in groups of 15 for interday results.

The data was processed separately for each molecule at each concentration, as the parameters are unrelated

2.3.1 Accuracy

Accuracy calculations are required in a validation study as they show how close to the actual sample concentration is to the obtained concentration. These calculations therefore identify discrepancies to be expected between the theoretical concentration and factual concentration. This is also called trueness. Thus, the analyst can gauge the range within which the obtained results lie when using a particular method. Accuracy mainly identifies if there are any losses or higher reading than expected. Also referred as bias, it was calculated by the following formula;

$$\text{Bias (\%) at concentration x} = \frac{\text{Grand mean of calculated concentration x - nominal concentration}}{\text{Nominal concentration}} \times 100$$

Where the grand mean of calculated concentration x is the mean of triplicate runs for intraday results, or the mean of all 15 values of the molecule at the given concentration. The nominal concentration is the concentration stated by the manufacturer for the specific molecule at the specific Level (i.e., Concentration).

2.3.2 Precision

Precision is described as the level of concordance between values, or how close the obtained values of measurement for the same theoretical value are to one another. This is needed to obtain a definite range within which the obtained value may lie, or rather, how close to each other are the output values to one another. Thus, it is a measure of constancy between the response measurements of the apparatus. This aids the analyst gauge the margin of error of the obtained readings.
For this standard deviation and coefficient of variation were calculated.

The standard deviation for intraday results was calculated using the formula for sample standard deviation, shown below:

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2},$$

Where

xi is one sample value

$\bar{x}$ is the sample mean

N is the sample size

While for interday results, the following formula was used;

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2}.$$

xi is an individual value

μ is the mean/expected value

N is the total number of values

the coefficient of variation was calculated using the following formula for intraday CV;

$$\text{Within Run CV (\%)} = \frac{\text{Standard deviation of a single run of samples}}{\text{Mean calculated value of a single run of samples}} \times 100$$

For interday CV the below formula was used instead;

$$\text{Between Run CV (\%)} = \frac{\text{Standard deviation of grand mean for each concentration}}{\text{Grand mean for each concentration}} \times 100$$

ANOVA calculations were run using MS Excel spreadsheets, grouping the repeats for a single day as one group, to a total of 5 groups. These were confirmed by a statistician using SPSS.

The Null Hypothesis states that no significant difference is observed between the mean result of each group; conversely the alternative hypothesis states that there is a significant difference between the mean of each group. The statistical test was run using an alpha value of 0.05. alpha value indicates the risk of a type I error, or a false positive; which in forensics may lead to misconduct of justice. This is the same a p-value, and the null hypothesis is rejected if the obtained value is below the threshold of significance, here being 0.05.

ANOVA is a parametric test, and was chosen as it performs well in skewed and nonnormal distributions as observed in the obtained data. ANOVA has satisfactory performance in groups having different spread for data (variance), and has high statistical power. The main reason for using this test was the fact that other parametric tests cannot compare more than 2 groups, and in the study, comparison was made between five separate sets of data. The test was performed to confirm or reject homogeneity, thus as per SWGTOX, non-parametric tests could not be used. Levene tests were carried out to confirm or regect homogeneity between groups

All calculations in section 2.3 were repeated for each solution concentration.
The results of the statistical calculations are presented in table form in Chapter 3 Results, each section outlining the results for a particular molecule. The Raw data is presented within Appendix 3.

Chapter 3

Results

3. Introduction

This chapter lists the results obtained from the tests carried out via the methodology described in Chapter 2. The first section details the statistical work carried out on each set of raw data, including the statistical equations used. The sections are grouped by methodology and control being used for the specific analysis.

In Appendix 3 are listed the raw data for each run, with averages worked out for each replicate pair in the first set of tables. It is the average that is used to work out accuracy and precision in this chapter, as it is a better approximation of the actual concentration detected by the apparatus.

The results for retention time derived from the preanalytical procedure for kit Code GC46010 used to obtain the concentration of cocaine, benzoylecgonine and cocaethylene are as follows; 11.865 min for benzoylecgonine, 11.849 min for cocaethylene and 11.578 MIN for cocaine. The internal standard had a retention time of 11.697 min.

The results for retention time derived from the preanalytical procedure for kit Code GC45010 used to obtain the concentration of morphine and 6-monoacetylmorphine are as follows; 12.819 min for morphine and 13.114 min for 6 monoacetyl morphine. The internal standard had a retention time of 13.362 min.

The statistical calculations carried out include bias (accuracy) and precision data listed by the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology (2013) as part of their validation requirements. The results were obtained using the following formulae:

$$\text{Bias (\%) at Concentration}_x = \frac{\text{Grand Mean of Calculated Concentration}_x\text{-Nominal Concentration}}{\text{Nominal Concentration}_x} \times 100$$

This was used to calculate within run and between run accuracy;

The coefficient of variation was calculated using the above equation, this is required for precision. More specifically, the below equations were used for in-run and between run CV.

$$\text{Within Run CV(\%)} = \frac{\text{Standard deviation of a single run of samples}}{\text{Mean calculated value of a single run of samples}} \times 100$$

$$\text{Between Run CV(\%)} = \frac{\text{Standard deviation of grand mean for each concentration}}{\text{Grand mean for each concentration}} \times 100$$

The ANOVA tests were carried out to either prove or reject homogeneity, no further statistical analysis was carried out as the ANOVA test displays which groups or values deviate from the others. As per SWGTOX (2013) this is the parametric test to be used for validation, other details are a derivation of this parametric test.

3.1. Results for benzoylecgonine and cocaethylene

In this section are displayed the accuracy bias calculations and precision studies for the Control Lyophilised solutions of benzoylecgonine level 1, benzoylecgonine level 2, cocaethylene level 1 and cocaethylene level 2, where Level 1 was the low concentration and Level 2 was of high concentration. These are grouped in subsections by control. The results in this section were obtained from tests carried out on controls Code CC43019 using the methodology described in subsection 2.2.2 and kit code GC46010, Controls kit and Methodology described in Chapter 2. Both benzoylecgonine and cocaethylene were present in the same controls, thus the results for both molecules were obtained simultaneously from each replicate, 15 separate replicates carried out in triplicate runs on 5 separate days.

The results in the following section were obtained using the calibration curves in Figure 3.1 and Figure 3.2 in Appendix 3.

The manufacturer's stated concentrations are listed in Table 2.1 in Appendix 2. These were used for the accuracy calculations.

3.1.1 Results for benzoylecgonine level 1

The average results obtained from the first and second readings listed in Table 3.1.1 were used in the calculations for bias and precision related to benzoylecgonine level 1. This is attached in Appendix 3. In Table 3.1.1 values for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. In Table 3.1.2 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. The ANOVA calculations in Table 3.1.3 were carried out to derive the f factor for the data, to prove or reject the null hypothesis stating: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level. In the test for homogeneity in Table 3.1.4 the Levene statistical test was used. The null hypothesis for this test stated that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.1.1: Bias calculation for benzoylecgonine control Level 1

Run	Mean ng/mL	Bias/accuracy
1 intraday result	75.37	25.62%
2 intraday result	82.93	38.22%
3 intraday result	88.85	48.08%
4 intraday result	75.42	25.70%
5 intraday result	89.46	49.10%
Interday results	82.44	37.40%

Table 3.1.2: Standard deviation and coefficient of variation for Precision study of benzoylecgonine control level 1

Run	Mean ng/mL	Standard deviation	Coefficient of variation %
intraday result Run 1	75.37	1.22	1.62
intraday result Run 2	82.93	6.41	7.73
intraday result Run 3	88.85	8.20	9.23
intraday result Run 4	75.42	9.55	12.66
intraday result Run 5	89.46	12.41	13.88
Interday results	82.44	10.06	12.20

Table 3.1.3 ANOVA Chart for benzoylecgonine control level 1

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	226.11	75.37	1.49
Run 2	3	249.30	83.10	41.10
Run 3	3	266.55	88.85	67.32
Run 4	3	226.26	75.42	91.14
Run 5	3	268.37	89.46	154.21

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	570.17	4	142.54	2.006163	0.169564	3.47805
Within Groups	710.52	10	71.05			
Total	1280.69	14				

Table 3.1.4: Test for homogeneity for benzoylecgonine control level 1

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	2.233	4	10	0.138
Based on Median	0.491	4	10	0.743
Based on Median and with adjusted df	0.491	4	4.905	0.745
Based on trimmed mean	2.045	4	10	0.164

3.1.2: Results for benzoylecgonine control level 2

The average results obtained from the first and second readings listed in Table 3.1.2 in Appendix 3 were used in the calculations for bias and precision related to benzoylecgonine level 2. In Table 3.1.5 values for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. In Table 3.1.6 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values.The ANOVA calculations in Table 3.1.7 were carried out to derive the f factor for the data, to prove or reject the null hypothesis stating: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level. In the test for homogeneity in Table 3.1.8 the Levene statistical test was used. The null hypothesis for this test stated that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.1.5: Bias calculation for benzoylecgonine control level 2

Run	mean	Bias/accuracy
intraday result Run 1	299.70	-0.10%
intraday result Run 2	273.32	-8.89%
intraday result Run 3	297.42	-0.86%
intraday result Run 4	254.67	-15.11%
intraday result Run 5	667.22	122.41%
Interday results	358.47	19.49%

Table 3.1.6: Standard deviation and coefficient of variation for Precision study of benzoylecgonine control level 2

Run	Mean microgram/mL	Standard deviation	Coefficient of variation %
intraday result Run 1	299.70	13.05	4.35
intraday result Run 2	273.32	40.99	15.00
intraday result Run 3	297.42	10.27	3.45
intraday result Run 4	254.67	5.36	2.10
intraday result Run 5	667.22	271.02	40.62
Interday results	358.47	184.84	51.56

Table 3.1.7: ANOVA Chart for benzoylecgonine control level 2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	899.10	299.70	170.29
Run 2	3	819.96	273.32	1680.40
Run 3	3	892.27	297.42	105.45
Run 4	3	764.00	254.67	28.73
Run 5	3	2001.67	667.22	73451.87

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	361603.7	4	90400.92	5.991836	0.010014	3.47805
Within Groups	150873.5	10	15087.35			
Total	512477.2	14				

Table 3.1.8 Test for homogeneity for benzoylecgonine control level 2

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	12.097	4	10	<.001
Based on Median	1.3	4	10	0.334
Based on Median and with adjusted df	1.3	4	2.058	0.475
Based on trimmed mean	10.21	4	10	0.001

3.1.3: Results for cocaethylene control level 1

The average results obtained from the first and second readings listed in Table 3.1.3, found in Appendix 3, were used in the calculations for bias and precision related to cocaethylene level 1. In Table 3.1.9 values for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. In Table 3.1.10 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. The ANOVA calculations in Table 3.1.11 were carried out to derive the f factor for the data, to prove or reject the null hypothesis stating: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level.In the test for homogeneity in Table 3.1.12 the Levene statistical test was used. The null hypothesis for this test stated that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.1.9: Bias calculation for cocaethylene level 1

Run	mean	Bias/accuracy
intraday result Run 1	71.67	2.39%
intraday result Run 2	74.98	7.19%
intraday result Run 3	82.53	17.91%
intraday result Run 4	71.12	1.60%
intraday result Run 5	79.28	13.25%
Interday results	75.98	8.56%

Table 3.1.10: Standard deviation and coefficient of variation for Precision study of cocaethylene control level 1

Run	Mean microgram/mL	Standard deviation	Coefficient of variation %
intraday result Run 1	71.67	4.36	6.09
intraday result Run 2	74.98	5.22	6.97
intraday result Run 3	82.53	2.77	3.36
intraday result Run 4	71.12	2.27	3.19
intraday result Run 5	79.28	7.99	10.08
Interday results	75.92	5.98	7.88

Table 3.1.11: ANOVA Chart for cocaethylene control level 1

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	215.01	71.67	19.03
Column 2	3	224.93	74.98	27.29
Column 3	3	247.60	82.53	7.69
Column 4	3	213.37	71.12	5.15
Column 5	3	237.83	79.28	63.85

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	290.99	4	72.75	2.9569	0.074863	3.47805
Within Groups	246.02	10	24.60			
Total	537.01	14				

Table 3.1.12: Test for homogeneity for cocaethylene control level 1

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	1.601	4	10	0.249
Based on Median	0.564	4	10	0.694
Based on Median and with adjusted df	0.564	4	6.144	0.698
Based on trimmed mean	1.508	4	10	0.272

3.1.4: Results for cocaethylene control level 2

The average results obtained from the first and second readings listed in Table 3.1.4, found in Appendix 3, were used in the calculations for bias and precision related to cocaethylene level 2. In Table 3.1.13 values for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. In Table 3.1.14 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. The ANOVA calculations in Table 3.1.15 were carried out to derive the f factor for the data, to prove or reject the null hypothesis which stated: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level.In the test for homogeneity in Table 3.1.16 the Levene statistical test was used. The null hypothesis for this test stated that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.1.13: Bias calculation for cocaethylene control level 2

Run	mean	Bias/accuracy
intraday result Run 1	311.68	3.89%
intraday result Run 2	337.16	12.39%
intraday result Run 3	339.75	13.25%
intraday result Run 4	323.60	7.87%
intraday result Run 5	355.63	18.54%
Interday results	333.57	11.19%

Table 3.1.14: Standard deviation and coefficient of variation for Precision study of cocaethylene control level 2

Run	Mean microgram/mL	Standard deviation	Coefficient of variation %
intraday result Run 1	311.68	8.59	2.76
intraday result Run 2	337.16	18.29	5.42
intraday result Run 3	339.75	7.24	2.13
intraday result Run 4	323.60	9.22	2.85
intraday result Run 5	355.63	10.92	3.07
Interday results	333.56	17.66	5.29

Table 3.1.15: ANOVA Chart for cocaethylene control level 2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	935.04	311.68	73.77
Run 2	3	1011.48	337.16	334.51
Run 3	3	1019.25	339.75	52.45
Run 4	3	970.81	323.60	85.06
Run 5	3	1066.88	355.63	119.29

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3348.49	4	837.12	6.293439	0.008504	3.47805
Within Groups	1330.15	10	133.01			
Total	4678.63	14				

Table 3.1.16: Test for homogeneity for cocaethylene control level 2

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	1.497	4	10	0.275
Based on Median	0.298	4	10	0.873
Based on Median and with adjusted df	0.298	4	5.259	0.868
Based on trimmed mean	1.359	4	10	0.315

3.2. Results for morphine and monoacetyl morphine

In this section are displayed the accuracy bias calculations and precision studies for morphine level 1, morphine level 2, monoacetyl morphine level 1 and monoacetylmorphine level 2. These are grouped by control. The results in this section were obtained from tests carried out on controls Code CC45019 and CC48019 using the methodology described in subsection 2.2.1 and kit code GC45010.

For this procedure the controls were used at 500 µL per test tube, with 500 µL from controls CC 45019 and 500 µL from controls code CC48019, thus the expected concentrations were as shown in Table 2.2, located in Appendix 2.

3.2.1: Results for morphine control level 1

The average results obtained from the first and second readings listed in Table 3.2.1 in Appendix 3 were used in the calculations for bias and precision related to morphine level 1. In Table 3.2.1 values for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values.In Table 3.2.2 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. The ANOVA calculations in Table 3.2.3 were carried out to derive the f factor for the data, to prove or reject the null hypothesis stating: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at

0.05 significance level. In Table 3.2.4 the test for homogeneity used was the Levene statistical test. The null hypothesis for this test stated that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.2.1: Bias calculation for morphine control level 1

Run	mean	Bias/accuracy
intraday result Run 1	108.07	8.07%
intraday result Run 2	121.53	21.53%
intraday result Run 3	117.89	17.89%
intraday result Run 4	118.06	18.06%
intraday result Run 5	132.95	32.95%
Interday results	119.70	19.70%

Table 3.2.2: Standard deviation and coefficient of variation for Precision study of morphine control level 1

Run	Mean microgram/mL	Standard deviation	Coefficient of variation %
intraday result Run 1	108.07	22.94	21.22
intraday result Run 2	121.53	3.48	2.86
intraday result Run 3	117.89	8.39	7.12
intraday result Run 4	118.07	2.83	2.39
intraday result Run 5	132.95	34.28	25.78
Interday results	119.70	17.40	14.54

Table 3.2.3: ANOVA Chart for morphine control level 1

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	324.20	108.07	526.1802
Run 2	3	364.58	121.53	12.09609
Run 3	3	353.67	117.89	70.37548
Run 4	3	354.21	118.07	7.980533
Run 5	3	398.85	132.95	1174.991

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	960.37	4	240.09	0.670043	0.627443	3.47805
Within Groups	3583.25	10	358.32			
Total	4543.62	14				

Table 3.2.4: Test for homogeneity for morphine control level 1

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	3.646	4	10	0.044
Based on Median	1.457	4	10	0.286
Based on Median and with adjusted df	1.457	4	4.497	0.35
Based on trimmed mean	3.469	4	10	0.05

3.2.2 Results for morphine level 2

The average results obtained from the first and second readings listed in Table 3.2.2, found in Appendix 3, were used in the calculations for bias and precision related to morphine level 2. In Table 3.2.5 values for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. In Table 3.2.6 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. The ANOVA calculations in Table 3.2.7 were carried out to derive the f factor for the data, to prove or reject the null hypothesis which stated: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level. In the test for homogeneity of Table 3.2.8 the Levene statistical test is used. The null hypothesis for this test stated that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.2.5: Bias calculation for morphine control level 2

Run	mean	Bias/accuracy
intraday result Run 1	389.22	29.74%
intraday result Run 2	436.03	45.34%
intraday result Run 3	435.12	45.04%
intraday result Run 4	392.83	30.94%
intraday result Run 5	580.28	93.43%
Interday results	446.70	48.90%

Table 3.2.6: Standard deviation and coefficient of variation for Precision study of morphine control level 2

Run	Mean microgram/mL	Standard deviation	Coefficient of variation %
intraday result Run 1	389.22	24.17	6.21
intraday result Run 2	436.03	45.13	10.35
intraday result Run 3	435.12	17.46	4.01
intraday result Run 4	392.83	13.21	3.36
intraday result Run 5	580.28	123.07	21.21
Interday results	446.70	85.40	19.12

Table 3.2.7: ANOVA Chart for morphine control level 2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	1167.67	389.22	584.25
Run 2	3	1308.10	436.03	2037.00
Run 3	3	1305.37	435.12	304.75
Run 4	3	1178.49	392.83	174.56
Run 5	3	1740.85	580.28	15146.13

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	72893.78	4	18223.44	4.99363	0.017903	3.47805
Within Groups	36493.38	10	3649.338			
Total	109387.20	14				

Table 3.2.8: Test for homogeneity for morphine control level 2

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	7.721	4	10	0.004
Based on Median	0.842	4	10	0.529
Based on Median and with adjusted df	0.842	4	2.534	0.59
Based on trimmed mean	6.566	4	10	0.007

3.2.3: Results for monoacetyl morphine control level 1

The average results obtained from the first and second readings listed in Table 3.2.3, found in Appendix 3, were used in the calculations for bias and precision related to 6-MAM level 1. In Table 3.2.9 values for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values.In Table 3.2.10 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values.The ANOVA calculations in Table 3.2.11 were carried out to derive the f factor for the data, to prove or reject the null hypothesis which stated: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level.In Table 3.2.12 the test for homogeneity used was the Levene statistical test. The null hypothesis for this test stated that the groups have equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.2.9: Bias calculation for 6 monoacetyl morphine control level 1

Run	mean	Bias/accuracy
intraday result Run 1	58.97	-26.29%
intraday result Run 2	56.70	-29.12%
intraday result Run 3	59.42	-25.72%
intraday result Run 4	58.14	-27.32%
intraday result Run 5	73.10	-8.63%
Interday results	61.26	-23.42%

Table 3.2.10: Standard deviation and coefficient of variation for Precision study of 6 monoacetyl morphine control level 1

Run	Mean microgram/mL	Standard deviation	Coefficient of variation %
intraday result Run 1	58.97	31.24	52.98
intraday result Run 2	56.70	12.93	22.81
intraday result Run 3	59.42	7.36	12.38
intraday result Run 4	58.14	4.90	8.44
intraday result Run 5	73.10	17.08	23.37
Interday results	61.26	15.41	25.16

Table 3.2.11: ANOVA Chart for 6 monoacetyl morphine control level 1

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	176.90	58.97	975.81
Run 2	3	170.10	56.70	167.24
Run 3	3	178.26	59.42	54.13
Run 4	3	174.42	58.14	24.06
Run 5	3	219.29	73.10	291.82

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	537.96	4	134.49	0.444428	0.774277	3.47805
Within Groups	3026.11	10	302.61			
Total	3564.07	14				

Table 3.2.12: Test for homogeneity for 6 monoacetyl morphine control level 1

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	3.843	4	10	0.038
Based on Median	0.608	4	10	0.666
Based on Median and with adjusted df	0.608	4	3.182	0.684
Based on trimmed mean	3.422	4	10	0.052

3.2.4: Results for 6 monoacetyl morphine control level 2

The average results obtained from the first and second readings listed in Table 3.2.4, found in Appendix 3, were used in the calculations for bias and precision related to 6-MAM level 2. In Table 3.2.13 calculations for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. In Table 3.2.14 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values.The ANOVA calculations in Table 3.2.15 were carried out to derive the f factor for the data, to prove or reject the null hypothesis which stated: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level.In the test for homogeneity in Table 3.2.16 the Levene statistical test was used. The null hypothesis for this test states that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.2.13: Bias calculation for 6 monoacetylmorphine control level 2

Run	mean	Bias/accuracy
intraday result Run 1	255.38	-14.87%
intraday result Run 2	245.46	-18.18%
intraday result Run 3	232.35	-22.55%
intraday result Run 4	235.56	-21.48%
intraday result Run 5	351.58	17.19%
Interday results	264.07	-11.98%

Table 3.2.14: Standard deviation and coefficient of variation for Precision study of 6 monoacetyl morphine control level 2

Run	Mean microgram/mL	Standard deviation	Coefficient of variation %
intraday result Run 1	255.38	17.48	6.85
intraday result Run 2	245.46	23.98	9.77
intraday result Run 3	232.35	10.87	4.68
intraday result Run 4	235.56	3.06	1.3
intraday result Run 5	351.58	77.39	22.01
Interday results	264.07	53.97	20.44

Table 3.2.15: ANOVA Chart for 6 monoacetyl morphine control level 2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	766.14	255.38	305.58
Run 2	3	736.38	245.46	575.18
Run 3	3	697.05	232.35	118.17
Run 4	3	706.69	235.56	9.38
Run 5	3	1054.74	351.5802	5989.35

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	29696.02	4	7424.01	5.304634	0.014833	3.47805
Within Groups	13995.32	10	1399.53			
Total	43691.34	14				

Table 3.2.16: Test for homogeneity for 6 monoacetyl morphine control level 2

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	2.663	4	10	0.095
Based on Median	2.375	4	10	0.122
Based on Median and with adjusted df	2.375	4	3.011	0.251
Based on trimmed mean	2.651	4	10	0.096

3.3. Results for cocaine

In this section are displayed the accuracy bias calculations and precision studies for cocaine level 1 and cocaine level 2. These are grouped by control. The results in this section were obtained from tests carried out on controls Code CC48019 using the methodology described in subsection 2.2.2 and kit code GC46010.

The manufacturer's stated concentrations are listed in Table 2.3, in Appendix 2. These were used for the accuracy calculations.

3.3.1 Results for cocaine level 1

The average results obtained from the first and second readings listed in Table 3.3.1 in Appendix 3 were used in the calculations for bias and precision related to cocaine level 1. In Table 3.3.1 the intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. In Table 3.3.2 values for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. The ANOVA calculations in Table 3.3.3 were carried out to derive the f factor for the data, to prove or reject the null hypothesis which stated: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level. In Table 3.3.4 the test for homogeneity used was the Levene

statistical test. The null hypothesis for this test stated that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.3.1: Bias calculation for cocaine control level 1

Run	Mean	Bias/accuracy
intraday result Run 1	106.29	51.84%
intraday result Run 2	85.51	22.16%
intraday result Run 3	70.40	0.58%
intraday result Run 4	132.15	88.78%
intraday result Run 5	431.62	516.60%
Interday result	146.17	108.81%
	Without run 5 98.59	Without run 5 40.84%

Table 3.3.2: Standard deviation and coefficient of variation for Precision study of cocaine control level 1

Run	mean	Standard deviation	Coefficient of variation
intraday result Run 1	106.29	55.44	52.16
intraday result Run 2	85.51	3.88	4.53
intraday result Run 3	70.40	2.82	4.00
intraday result Run 4	132.15	113.62	85.98
intraday result Run 5	431.62	596.33	138.16
Interday result	146.17	204.28	139.76
	Without run 5 98.59	Without run 5 56.62	57.42

Table 3.3.3: ANOVA Chart for cocaine control level 1

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	318.88	106.29	3073.77
Run 2	3	256.54	85.51	15.03
Run 3	3	211.22	70.41	7.92
Run 4	3	396.45	132.15	12908.71
Run 5	3	863.24	287.75	239905.30

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	92325.63	4	23081.41	0.45	0.77	3.48
Within Groups	511821.50	10	51182.15			
Total	604147.2	14				

Table 3.3.4: Test for homogeneity for cocaine control level 1

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	13.067	4	10	<.001
Based on Median	0.846	4	10	0.527
Based on Median and with adjusted df	0.846	4	2.268	0.596
Based on trimmed mean	10.335	4	10	0.001

3.3.2 Results for cocaine level 2

The average results obtained from the first and second readings listed in Table 3.3.2 in Appendix 3 were used in the calculations for bias and precision related to cocaine level 2. In Table 3.3.5 values for intraday and interday accuracy levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. Table 3.3.6 summarises the calculations for intraday and interday standard deviation and coefficient of variation levels were calculated using the average concentration of each run. For intraday bias the triplicates for the daily runs were used, while interday results were calculated using all 15 values. Table 3.3.7 shows the ANOVA calculations carried out to derive the f factor for the data, to prove or reject the null hypothesis which stated: there was no significant difference between the obtained data and the expected values for accuracy, thus the values were within an expected range of precision. Alpha value was set at 0.05 significance level. Table 3.3.8 is the test for homogeneity, the Levene statistical test is used. The null hypothesis for this test stated that the groups had equal variances. The assumption of homogeneity between groups was met if the p value was greater than 0.05.

Table 3.3.5: Bias calculation for cocaine control level 2

Run	Mean	Bias/accuracy
intraday result Run 1	338.11	12.70%
intraday result Run 2	260.98	-13.01%
intraday result Run 3	291.94	-2.69%
intraday result Run 4	248.50	-17.17%
intraday result Run 5	534.03	78.01%
Total	334.71	11.31%
		Without run 512.73%

Table 3.3.6: Standard deviation and coefficient of variation for Precision study of cocaine control level 2

	mean	Standard deviation	Coefficient of variation
intraday result Run 1	338.11	86.74	25.65
intraday result Run 2	260.98	19.14	7.33
intraday result Run 3	291.94	63.31	21.69
intraday result Run 4	248.50	15.36	6.18
intraday result Run 5	534.03	648.30	124.40%
Interday result	334.71	261.8096	78.29
	Without run 5 284.8808	56.71698	18.91%

Table 3.3.7: ANOVA Chart for cocaine control level 2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Run 1	3	1014.32	338.11	7523.29
Run 2	3	782.92	260.97	366.18
Run 3	3	875.83	291.94	4008.36
Run 4	3	745.50	248.50	236.06
Run 5	3	1602.08	534.03	420292.90

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	163310.8	4	40827.69	0.47	0.76	3.48
Within Groups	864853.5	10	86485.35			
Total	1028164	14				

Table 3.3.8: Test for homogeneity for cocaine control level 2

Tests of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Based on Mean	13.493	4	10	<.001
Based on Median	0.881	4	10	0.509
Based on Median and with adjusted df	0.881	4	2.07	0.59
Based on trimmed mean	10.712	4	10	0.001

3.4: Tests on urine samples obtained from patients.

This subsection lists the results obtained for the concentration of cocaine, benzoylecgonine, cocaethylene, morphine and 6-monoacetyl morphine. This was performed to confirm the apparatus can detect the molecules of interest within a matrix obtained from an uncontrolled source, which can be contaminated by various molecules, all of which can influence sensitivity.

Table 3.4.1 shows the obtained concentrations for cocaine benzoylecgonine and cocaethylene in the samples tested on 19th October 2023. These were obtained using the calibration curves in Appendix 3.

Table 3.4.1: Concentration of urine samples tested for cocaine and its metabolites

Sample	Cocaine ng/mL	Benzoylecgonine ng/mL	Cocaethylene ng/mL
A	Below calibration	Not detected	Below calibration
B	Below calibration	505.516	Not detected
C	5593.897	121948.146	Not detected

Table 3.4.2 shows the concentration of morphine and 6 monoacetyl morphine present in the samples tested on 25th October 2023. These were obtained using the calibration curves in Appendix 3.

Table 3.4.2: Concentration of urine samples tested for opiates.

Sample	Morphine ng/mL	6 monoacetyl morphine ng/mL
A	14806.955	Not detected
B	1949.257	Not detected
C	250.640	34.556

3.5: Conclusion

The results listed in this chapter were discussed in detail in Chapter 4. The Discussion opens with an overview of the obtained results and their initial interpretation, followed by a thorough discussion in relation to retrieved literature. Any shortcomings and suggestions for improvement are mentioned, with a conclusion wrapping up the obtained data.

Chapter 4

Discussion

4.1 Discussion

This section includes a thorough overview of the obtained results, discussing main findings and their implications. This also includes any possible reasons for any unusual results. This is followed by a list of shortcomings and suggestions for improvement, and a conclusion wrapping up the study. In this study no test for outliers was carried out, however the obtained results were compared both to the manufacturer's ranges and the intra and interday median values for each molecule.

4.2 Discussion of results in Section 3.1

General overview of raw data and precision

In this section the results obtained with respect to the validation of the methods for the determination of benzoylecgonine level 1, benzoylecgonine level 2, cocaethylene level 1 and cocaethylene level 2. are discussed. These results are grouped according to the control. The results in this section were obtained from tests carried out on controls Code CC43019 using the methodology described in subsection 2.2.2 and kit code GC46010. The sections are named according to the control results being discussed.

The values obtained for precision calculations show a high degree of variability, with raw data ranging from 66.85 to 97.85 ng/mL for benzoylecgonine level 1; from 234.98 to 978.08 ng/mL for benzoylecgonine level 2; from 67.04 to 86.48 ng/mL for cocaethylene level 1; from 301.95 to 367.17 ng/mL for cocaethylene level 2. These ranges were obtained from the average reading of each test sample. Each sample

prepared was run twice on the GC/MS, with most preparations showing significant difference between first and second reading, as observed in the results section.

This may indicate there are some issues with the system, suggesting it may require recalibration or further maintenance as if is unusual for the same sample to present drastically different results. There were some instances where the values obtained were very similar between the first and second reading of each sample, with the lowest difference being; 0.37 ng/mL for BEG level1; 3.70 ng/mL for BEG level 2; 0.48 ng/mL for cocaethylene level 1; 0.18 ng/mL for cocaethylene level 2. Thus, it can be assumed the system is capable of a high degree of reproducibility within a narrow margin of error but fails to do so consistently.

The variability of the replicates within run was also significant, even when considering the averages of first and second readings. The coefficient of variation should not exceed 10%, and ideally is below 5% (SWGTOX, 2013), however many values for CV exceeded the 10% threshold. This indicated the method needs improvement to have reproducible results, again, considering that within run replicates were prepared from the same homogenous sample in many cases, the possibility of differences within the test solution being the cause of such discrepancy is minimal; with either user error during preparation or system errors during processing in the GC/MS having been major sources of error. The variability between readings for the same run indicated that the system may need to be rechecked.

4.2.1 Benzoylecgonine level 1

For BEG, the obtained range of values differs by approximately 31 ng/mL, which was slightly higher than the manufacturer's range, at 24 ng/mL. This was mainly due to the lowest value being at 66.85 ng/mL. In comparison, the next lowest value was at 73.70 ng/mL, suggesting the low reading was an outlier. However, the range obtained experimentally was higher than expected, as all values were above the nominal value of 60 ng/mL. One theory is the apparatus could be sensitive to other components of the control solutions. The controls for BEG also contain coca ethylene, several amphetamines, ketamine, methadone, and ephedrines. These compounds could have interacted with the readings, slightly skewing the results. Another plausible reason could be the calibrators at the lower end of the curve had slightly lower concentrations than indicated, skewing the obtained value for the test controls. The latter is more unlikely as linearity at 0.991 was obtained, so any skew in the calibration curve should not produce drastic shifts in results.

4.2.2 Benzoylecgonine level 2

For level 2, the range was from 234.98 ng/mL to 978.08 ng/mL. The highest value was a clear outlier, with all values obtained from the same run showing a drastic difference from the others, suggesting all readings from this run were out of range. The range obtained is much higher than that indicated by the manufacturer, which was between 255 ng/mL and 345 ng/mL. When not considering the values obtained on Run 5, suspected to be erroneous, 316.53 ng/mL is the highest value; giving a range between 234.98 ng/mL and 316.53 ng/mL, which was only slightly lower than that given by the manufacturer. The values for the last run were suspected to have been due to a system failure, due to the drastic difference between these results and those obtained from the other four runs; or, a significant contamination from previous runs, since there was a significant gap in obtained results even amongst values for the 5th run considered separately.

Calibration curve linearity value was equal to 0.997, which is very accurate, thus the calibration curve linearity excluded to have impacted the obtained results.

4.2.3 Test for homogeneity for benzoylecgonine

For benzoylecgonine level 1 the Levene statistics values were all over 0.05. These results show there is homogeneity in the obtained results. However, the results for benzoylecgonine control level 2 have a p value <0.001 based on the mean value and 0.001 based on the mean trimmed value. Thus, for this data set the null hypothesis (i.e., That there was no statistical difference between means) cannot be accepted. In fact, run 5 has a mean of 667.22 ng/mL, due to all samples within this run being out of range. The p value obtained using the median was over 0.05, confirming run 5 was the main reason for deviation.

4.2.4 Cocaethylene level 1

The range obtained for cocaethylene level 1 was from 67.04 ng/mL to 86.48 ng/mL, which was slightly higher than the manufacturer's, which stands between 56 ng/mL and 84 ng/mL. The variability was lower, at only 19.47 compared to the manufacturer's 28 ng/mL margin, showing the methodology was robust for this analyte at low concentrations. The obtained results were slightly high compared to the nominal 70 ng/mL, but the skew was lower than that observed for BEG, which indicated the confounder for BEG was not related to sample preparation or other analytes within the controls.

4.2.5 Cocaethylene level 2

The range for cocaethylene level 2 was between 301.95 ng/mL and 367.17 ng/mL, slightly higher than the manufacturer's, which was between 255 ng/mL and 345 ng/mL. The range difference was lower for the experimental, at 65.23 ng/mL; than for the manufacturer's values, which showed a 90 ng/mL difference. The values obtained on run 5 were the highest also for cocaethylene. BEG and cocaethylene were tested via the same run samples, however cocaethylene level 2 values for run 5 did not show a large enough difference from the manufacturer's own to suggest major procedural flaws. Even so, run 5 for cocaethylene also showed higher values than the other runs, and were the only values which overshot the manufacturer's highest range value, albeit slightly. The difference does not warrant considering the elimination of run 5 values from parameter calculations.

4.2.6 Bias/Accuracy

The obtained values for bias are varied, with some failing to meet the =/- 20% threshold stated by SWGTOX parameters. The runs for benzoylecgonine level 1 failed to meet the criteria, at 37.40% bias. This was because the obtained values were consistently above the manufacturer's given concentration, which was 60 ng/mL. In contrast, all mean run values exceeded 70 ng/mL. The values show concordance, suggesting the system is consistently over detecting BEG at lower concentrations. This was not the case for benzoylecgonine level 2, which gave an overall accuracy of 19.49%, which was within parameters. It was noted, however, that run 5 for this molecule and concentration had readings which were grossly out of range, suggesting

a malfunction. The value calculated considering the out-of-range values had a positive bias, whereas bias readings for separate runs 1 to 4 all had a negative bias, also below 20%. This suggests there was a slight deviance from the manufacturer's value at high concentrations.

Both cocaethylene level 1 and 2 had bias within the expected threshold, at 8.56% and 11.19%, respectively. This strongly indicated that the method is robust for this analyte, as the resultant bias was well under the maximum accepted 20%. Both values had a positive skew, however, which may be due to a slight over dilution of the calibrators.

4.2.7 Precision via Anova

For both molecules, Anova results showed an f value below the F-Critical value, thus the null hypothesis was accepted, and the observed differences were not significant at an alpha value of 0.05. This means the method was precise within acceptable levels. However, all high concentration analyses gave an F value above the critical value, showing the precision was not high enough.

Precision should increase with increasing concentration, as the system should detect higher concentrations of any given molecule with more efficiency. However, the variability of level 2 controls was higher than level 1 controls for all tested molecules. Increased variability in level 2 controls as compared to level 1 controls is also observed in the range of differences between first and second reading of single test replicates.

4.2.8 Test for homogeneity

For benzoylecgonine level 1 the Levene statistics values were all over 0.05. These results show there is homogeneity in the obtained results. However, the results for benzoylecgonine control level 2 have a p value <0.001 based on the mean value and 0.001 based on the mean trimmed value. Thus, for this data set the null hypothesis (i.e., That there was no statistical difference between means) could not be accepted. In fact, run 5 has a mean of 667.22 ng/mL, as all samples within this run were out of range. The p value obtained from the median was over 0.05, confirming run 5 was the main reason for deviation.

For cocaethylene level 1 the Levene statistical results were all over 0.05, homogeneity was confirmed. This was also the case for cocaethylene controls level 2, with p value exceeding 0.05 using both mean and median. Thus, the null hypothesis of the test was accepted and the means of all groups were not statistically different from each other for both level 1 and 2, and any observed differences were due to random occurrence.

Only benzoylecgonine showed variability, albeit both molecules being present within the same mixture. This could imply that the error was not caused by the system or operator, but could have been a manufacture defect. Only run 5 for level 2 displayed this error, making this theory plausible. The controls are mass produced, so errors may go undetected in single vials.

4.3 Discussion of results in section 3.2

General overview

This Section discussed the results obtained from controls Code CC45019 and CC48019 with the Methodology described in Subsection 2.2.1 and kit code GC45010. These were namely the test results for morphine level 1, morphine level 2, 6 monoacetylmorphine level 1 and 6 monoacetylmorphine level 2, grouped by control in this discussion.

When discussing the results for morphine and 6-MAM it must be noted that only 500 µL of the respective solutions was used during the procedure, resulting in readings being half of what is expected by the manufacturer's data, thus the obtained values will be compared to the manufacturer's values all halved accordingly as shown in the below table 4.3.

Table 4.3: Calibrator concentration for opiates

control	Manufacturer concentration ng/mL	Actual expected concentration ng/mL	Manufacturer range ng/mL	Expected range ng/mL
Morphine level 1	200	100	160-240	80-120
Morphine level 2	600	300	510-690	255-345
6-MAM level 1	160	80	128-192	64-96
6-MAM level 2	600	300	510-690	255-345

The values obtained for precision calculations showed a high degree of variability, with raw data ranging from 81.85 to 168.96 ng/mL for morphine level 1; from 365.70 to

663.48 ng/mL for morphine level 2; from 23.08 to 89.11 ng/mL for 6-MAM level 1, and; from 219.84 to 428.07 ng/mL for 6-MAM level 2. These ranges were obtained from the average reading of each test sample. Each sample prepared was run twice on the GC/MS, most preparations showed significant difference between first and second reading, as observed in the results section. This may indicate there are some issues with the system, suggesting it may require recalibration or further maintenance as it is unusual for the same sample to present drastically different results. There were some instances where the values obtained were very similar between the first and second reading of each sample, with the lowest difference values being 3.03 ng/mL for morphine level 1; 0.02 ng/mL for morphine level 2; 0.28 ng/mL for 6-MAM level 1, and; 1.91 ng/mL for 6-MAM level 2. This shows the system was capable of reproducibility within a narrow margin of difference, however it failed to achieve this consistently.

The variability of the replicates within run was also significant, even when taken from the averages of first and second readings. The coefficient of variation should not exceed 10%, and ideally is below 5% (SWGTOX, 2013), however many values for CV exceeded the 10% threshold. This indicates the method needed improvement to have reproducible results. Again, within run replicates were prepared from the same homogenous sample in many cases, so the possibility of differences within the test solution having been the cause of such discrepancy was minimal; with either user error during preparation or system errors during processing in the GC/MS having been major sources of error. The variability between readings for the same run indicated that the system may have needed to be rechecked.

The low concentrations analysed may have resulted in reduced precision for morphine and 6-MAM. These two analytes were mixed in a 1:1 ratio for each run of both calibrators and controls, with the resulting actual concentration of the run solutions being half that listed. It is worth considering that lower concentrations may have resulted in reduced accuracy by the system when detecting sample concentration, however this was suggested by the provided methodology and the calibration curves were obtained via the same procedure. The correlation of the values of the calibration curve was within the desired linearity, thus the likelihood of errors derived from calibration curve inaccuracy was reduced. The calibration curves were prepared using a blank and 4 different concentrations for each analyte, with each level being tested twice and the average being used in calibration curve derivation. This further reduced source of error. Pipetting errors were also more likely with smaller volumes being used.

4.3.1 Morphine Level 1

Morphine level 1 had an overall average value of 119.70 ng/mL, which borders the manufacturer's maximum concentration, and the concentrations obtained range from 81.85 ng/mL to 168.96 ng/mL, as opposed to the manufacturer's 80 ng/mL to 120 ng/mL. The obtained maximum of 168.96 ng/mL is an outlier, as were 3 other values; but the other outliers did not go beyond 130 ng/mL, so the maximum could have been a system or procedural failure. The second highest value is 129.17 ng/mL, which overshot the manufacturer's maximum by over 9 ng/mL.

4.3.2 Morphine level 2

Morphine level 2 had an average value of 446.70 ng/mL, with values ranging between 365.70 ng/mL - 663.48 ng/mL, with a difference of over 300ng/mL. This contrasted with the manufacturer's range, at a 90 ng/mL difference. Values 2b and 2c from 8th August were outliers as the next highest value was 486.61 ng/mL, almost 200 ng/mL lower than the maximum obtained value. This value was still much higher than the manufacturer's maximum.

There were multiple reasons which could have contributed to the difference between the expected and actual obtained results. The use of 500 µL in sample preparation could have introduced slight inaccuracies due to pipetting errors. Another more significant contributor to error could have been the control itself. The control for morphine also contained codeine, dihydrocodeine and ethylmorphine. These

molecules all have a similar structure to morphine, and thus could have altered the obtained results. Of relevance, was a qualifier peak of 234, common to both codeine and morphine in the manufacturer's data. One assumption is that this peak was enhanced by the presence of codeine, thus gave values which were higher than expected.

4.3.3 6 Monoacetyl morphine level 1

For 6-MAM the mean value was 61.26 ng/mL, almost 20 ng/mL lower than the manufacturer's average. This was congruent with the obtained range, which was between 23.08 ng/mL and 89.11 ng/mL, as opposed to 64 to 96 stated by the manufacturer. The variability was also higher for the obtained results, at 66.05; with the manufacturer stating a difference of 32 ng/mL. Of 15 values, 9 were below the manufacturer's declared minimum, suggesting the system fails to accurately quantify 6-MAM at low concentrations. The procedure involves a 3-hour enzyme digestion process, which could have adversely affected 6-MAM concentrations.

4.3.4 6 Monoacetyl morphine level 2

The mean obtained value for 6-MAM level 2 was 264.07 ng/mL, slightly lower than the manufacturer's stated 300 ng/mL. The values ranged between 219.84 ng/mL and 428.07 ng/mL, with almost 200 ng/mL difference between the minimum and maximum value. The maximum was an outlier, with the next highest value of 353.35 ng/mL also being a suspected outlier, as the third highest is over 75 ng/mL lower at 275.14 ng/mL. The range given by the manufacturer was much more stringent than the obtained, being 255 ng/mL to 345 ng/mL. However, when the two highest values are considered outliers the variability drops drastically, as 219.84 ng/mL and 275.14 ng/mL gave a narrower range than the manufacturer. This would also give a range that runs at lower concentrations to the manufacturer's, in a similar pattern to what was observed in level 1. The difference was less pronounced at higher concentration as be expected, but was not overseen. One possibility for the low results is that 6-monoacetyl morphine degraded during the sample processing time itself. According to Sitasuwan, Melendez et al. (2015), 6-MAM is predisposed to deacetylation in acidic environments. The kit uses enzymatic hydrolysis to circumvent this issue as much as possible, but the buffer used still may be impacting 6-monoacetylmorphine recovery. According to Sitasuwan, Melendez et al. (2019), some enzymes used for opiate hydrolysis can also deacetylate 6-monoacetyl morphine due to the impurities within the preparation. It is unlikely that other solvents are of significant impact to the molecule's concentration, as the processing times within the columns are very short.

The procedure was carried out as instructed by the manufacturer, however the results for the opiates test were not of a satisfying accuracy level. The procedure can also be

carried out for morphine and 6-MAM separately, using 1 mL of a single solution. This approach may produce much more accurate results. It should be considered, however, that in forensic analysis lower concentrations are more common than high, thus low performance at lower concentrations indicates methodology should be improved. The possibility of other components of the calibrators having interacted should be considered, as actual urine samples are probable to have more than one substance dissolved.

4.3.5 Bias/Accuracy

The obtained values for bias were varied, some have failed to meet the =/- 20% threshold stated by SWGTOX parameters. Morphine level 1 showed bias levels within threshold, at 19.70% on average. It must be noted, however, that run 2 and 5 exceeded the threshold individually, suggesting the system may be overreading this analyte. The bias for morphine level 2 was 48.90%, well out of the accepted range (up to 20%). This suggests the system could be over estimating this parameter. Such an occurrence may be due to the presence of other opiates in the mixture analysed. This could have been improved by deriving calibration curves for the other opiates and comparing the results to each of these for more accurate estimates.

The bias of 6-MAM level 1 exceeded the excepted threshold, at 23.42%, with readings consistently below the manufacturer's stated concentration for this molecule. This indicated the apparatus was less sensitive than required to this analyte at lower concentrations. In contrast, 6-MAM level 2 was within parameters, at -11.98% bias,

with individual runs 3 and 4 slightly overshooting at 21.48% and 22.55% respectively. This indicates the system was sufficiently sensitive to 6-MAM at high concentrations. Values still were in the lower part of the provided range, indicating there could be some losses.

4.3.6 Test for homogeneity for morphine and 6-monoacetyl morphine

Morphine had particular results for level 1, with the Levene statistic using the mean just below 0.05, at 0.044, but within range using the median and exactly 0.05 using a trimmed mean. This indicates results are dubious, with run 5 showing the most variance. Level 2 showed clear variance when using the mean, with a p value of 0.004 and 0.007 for the trimmed mean. Using the median, the results had a p value exceeding 0.05, indicating run 5 as outliers, as run 5 is consistently showing discrepancies. Thus, the method is fairly consistent, and any differences could be due to operator errors.

Monoacetyl morphine displayed borderline results for homogeneity, with the Levene statistic calculated from the mean of 0.038, which was below the minimum of 0.05, and 0.052 when calculated from a trimmed mean. The values were above 0.05 when calculated from the median, which indicated there was an outlier. For this molecule, run 1 showed the most variance, but run 5 was the one with the most diverging mean, thus run 5 was the outlier in the test for homogeneity. Monoacetyl morphine level 2 displayed a similar pattern, but gave a p value greater than 0.05 using both mean and median. The median still was significantly higher, with the mean for group 5 as a

possible outlier amongst the others, but this had no impact on the test for homogeneity.

As morphine and monoacetyl morphine were both tested within simultaneous runs, and in both level 1 and 2 for each of the molecules run 5 displayed a higher mean concentration; it was a procedural error, most probably of lyophilised solution reconstitution, which produced incongruent results.

4.4 Discussion of results in Section 3.3

This Section will discuss the results obtained from controls Code CC48019 processed with the Methodology described in Subsection 2.2.2 and kit code GC46010. These were the test results for, cocaine level 1 and cocaine level 2, grouped by control in this section.

4.4.1 Cocaine level 1

The mean obtained value for cocaine level 1 was 146.17 ng/mL, which was highly incongruent with the manufacturer's stated average of 70 ng/mL. The values obtained on run 5 were out of range, thus another mean was calculated excluding these values. The mean obtained from runs 1 to 4 (excluding run 5) was 98.59 ng/mL, which still falls outside the manufacturer's range, from 56.0 ng/mL to 84.0 ng/mL. This is because many values were overshot from the maximum indicated concentration, the highest value (excluding run 5) was 263.34 ng/mL and the second highest 170.29 ng/mL. The outlier values were suspected to be due to pipetting errors, mainly of the internal standard. Run 5 showed a prominent peak in all repeats at an unexpected point, thus there could have been some contaminant. As the peak was close to the peaks used for quantitation this could have influenced the final reading. The impurities could have originated from the test tubes in which the procedure was carried out.

4.4.2 Cocaine level 2

The mean value for cocaine level 2 controls was 334.71 ng/mL. For this concentration run 5 was also out of range, thus a mean was derived excluding these values. The mean excluding run 5 was 284.88 ng/mL. Both values were within the manufacturer's given range, which was 255.00 ng/mL to 345.00 ng/mL, with a lower average being observed when eliminating run 5. Another value which was above the range was in group 1, at 436.24 ng/mL, and 363.50 ng/mL in run 3. Run 2 had a result below range, at 240.93 ng/mL, excluding run 5 and the two highest values suspected to be out of range, the obtained results were generally low. Run 3 also had a below range reading and run 4 had two readings lower than 255 ng/mL. This indicated there could have been procedural errors of reconstitution at higher concentrations of the calibrators, off-putting the curve to lower concentrations. Run 5 was out of range, again suspected to be a pipetting error of the internal standard solution during sample preparation or contamination of the samples from improperly cleaned test tubes. The abnormal peak observed in level 1 was also observed for level 2 run 5, which again could have influenced the MS quantitation.

4.4.3 Bias/ Accuracy

For control level 1 only run 3 was within the 20% bias range at 0.58%, with run 2 being slightly overshot at 22.16% and the others being grossly out of range. This was expected as all other groups had values which were out of range. For cocaine controls level 2, only run 5 was out of the expected accuracy level, all others having below 20% discrepancy, showing the method is consistent at the higher end of quantitation. The interday result for accuracy was out of range both when including and excluding run 5 for cocaine, suggesting the method was not adequately implemented at lower concentrations. For cocaine controls level 2 the accuracy threshold was reached for interday results, thus it was assumed that any out-of-range results for this concentration were random errors. The lack of accuracy at lower concentrations could have resulted from incorrect control preparation at this concentration, which had a more pronounced effect at low concentration, or errors with the internal standard. The internal standard could have mixed incompletely, resulting in inaccurate quantitation.

For the precision study, only run 2 and 3 had a coefficient of variation lower than 10% for controls level 1; and runs 2 and 4 for controls level 2. This indicates the system is not quantitating the molecule within desired precision levels.

4.4.4 Anova charts

For both control levels, the obtained p value was below 0.05. thus, the null hypothesis is rejected and it was assumed there is statistical difference between the means of the groups. This implies that any errors present in method implementation were significant to the methodology.

4.4.5 Further observations of results

Accuracy was also lower than expected for all analytes at both high and low concentration. Many of the lower concentrations gave a slightly high reading, whereas the high concentrations gave low readings. It is worth noting that the ranges of concentration readings for all substances at both levels for each analyte provided by the manufacturer are also wide, thus the variability is to be expected on a general level. This study was relatively short, and used a small quantity of replicates. This procedure could partially be the reason for the resulting observed lack of precision, but does not justify the reduced accuracy. Increasing the quantity of samples within each run could potentially increase accuracy levels, while also indicating more clearly if the out-of-range results obtained were a system error or more dependent on the operator. In larger groups such differences would also have less impact on the accuracy and precision values.

One reason for lower-than-expected readings are losses. Losses can result from physical shortcomings such as incomplete sample transfer, or chemical interactions resulting from the exposure to various solvents or degradation from temperature changes and exposure to gases. Over dilution of the controls could have occurred,

resulting in actual lower concentration; and, under dilution of the calibrators resulting in skews within the calibration curves. These factors could have influenced the final concentration detected by the GC/MS. These, however, fail to explain why a few readings had significantly higher concentrations than expected; which alludes once more to a system error. Errors in internal standard preparation and pipetting could also produce such massive inaccuracies. Some readings were out of range, skewing the calculations. Loss of sample in the method is accepted up to 20% in forensics (SWGTOX, 2013), and this should be calculated accordingly to be accounted for when presenting the result. This is to ensure that conclusions are being drawn on factual values, as excessively low concentrations may result in a false conclusion that the drug did not cause death. By the same principle, when the obtained reading is falsely high, it may indicate overdose when it was not the primary cause of death.

When a large difference was observed between the first and second reading the obtained values showed increased variability from the trend, indicating that such cases were erroneous readings. This predisposition causes problems, as the method should function accordingly within all the range, and not result in dubious results. For forensic drug analysis the typical concentrations are very low, as the urine samples are obtained from autopsies of people dead of overdose in most cases. Thus, the sample is already significantly degraded by the time it reaches the laboratory.

Methyl ecgonidine and ecgonidine may form as artefacts within the GC-MS system (Shimomura, Hodge et al., 2001). These may influence the final mass spectrum and the

quantitation. This could be the source of the unusual peak observed in the cocaine spectrum. Chericoni, Stefanelli et al (2015) managed to obtain much higher accuracy and precision levels, below 15% difference for cocaine, cocaethylene and benzoylecgonine. Rees, McLaughlin et al. (2012) had similar accuracy and precision with cocaine and benzoylecgonine. Alvear, von Bear et al. (2014), This indicates both parameters could be improved.

Bevalot, Bottinelli et al. (2014) obtained accuracy and precision of around 10% and lower both for cocaine and morphine and 6 monoacetylmorphine; Smith, Nickols et al. (2014) have similar results, with the study including other opiates. This shows the method should be able to quantify morphine and 6 monoacetyl morphine within the desired accuracy and precision parameters.

4.4.6 Precision via Anova

For all molecules, Anova results showed an f value below the F-Critical value, thus accepting the null hypothesis that the observed differences are not significant at an alpha value of 0.05. this means the method is precise within acceptable levels. However, all high concentration analyses gave an F value above the critical value, showing the precision is not high enough.

Precision should increase with increasing concentration, as the system should detect higher concentrations of any given molecule with more efficiency. However, the variability of level 2 controls is higher than level 1 controls for all tested molecules, as seen in the results section. Increased variability in level 2 controls as compared to level 1 controls is also observed in the range of differences between first and second reading of single test replicates.

4.5 Urine Sample Tests

For cocaine and its metabolites, a common pattern was observed for the urine samples. Cocaethylene was not detected in samples B and C and below quantitation for sample A. As cocaethylene was detected in sample A it was unlikely to be lack of sensitivity to the analyte in the system. In sample A cocaine was detected in small quantities, while benzoylecgonine was absent. It was possible the sample was obtained from someone who just recently had the drug, thus the quantities of cocaine present in urine were low. Cocaine and its metabolites are excreted in urine, thus must

first undergo absorption and distribution for cocaine, and metabolism must occur for any metabolite to be present. Cocaethylene is a minor metabolite of cocaine, and one which forms in the presence of ethanol by transesterification in the liver (Redinbo, Bencharit et al., 2003); thus, it is possible no cocaethylene forms when the drug is taken. Cocaine was detected below LoQ for samples A and B while it could be quantitated for sample C. This can occur as cocaine is metabolized to benzoylecgonine prior to being excreted in urine and obtaining the sample. Sample C contained high quantities of the molecule, but still at a ratio approximately 1:22 cocaine to benzoylecgonine. Benzoylecgonine was detected in samples B and C. This shows the system can identify all molecules within a true urine sample.

For opiates morphine was identified in all samples, with 6 monoacetyl morphine being detected only in sample C. This can be explained as 6 MAM converts quickly to morphine, thus was less likely to be present in true samples. Another explanation could be the samples were not obtained from someone who took heroin; thus no 6-MAM could be present. Since the samples were of unknown origin due to anonymity the context from which the sample was taken was unknown, thus no clear inference could be done on the absence of 6 MAM. The system could identify both analytes in a true urine matrix, even when present together, showing the method was sensitive to both molecules.

4.6 Limitations of the study

The methodology presented had several drawbacks. The study was relatively short, and comprised of a small number of replicates. This resulted in statistical analysis which showed a high degree of variability, which may not be significant with larger numbers of test samples.

Another problem was the relative time between each run. There were instances where runs for the same analyte were performed even months away from each other. This could have affected stability due to slight differences in ambient temperature (this was controlled via air conditioning, but movement between one area and another could influence via temperature fluctuations). Time between runs could also have influenced the stability of any frozen previously prepared reagents. No reagent exceeded the suggested storage time in the freezer already reconstituted, but when stored for a long time some degradation could have occurred.

The kits themselves also varied in purchase times, with initial kits having been used for a significant time, but new ones being bought as soon as the previous were used up. This could have slightly altered the stability of reagents. Batch to batch variability could have influenced the results.

Half way through the replicate tests a fault in the filament occurred, and thus the filament had to be changed. This led to new calibration curves being derived, after the runs were initiated. This could have resulted in system inaccuracies.

The lyophilised controls all contained more than one compound for analysis, which could have contributed to the erroneous results of certain tests. This, however, would imply there was significant cross-reactivity and the method was not reliable.

The methodology was used as indicated by the manufacturer, but some settings may have failed to work as the manufacturer intended.

The opiate test included enzymatic workup, which could have influenced the seemingly low readings. One assumption was that the lysis of the glucuronides was not completed within the suggested timeframe. This could be tested by carrying out the study and using different timeframes for hydrolysis to optimise derivatisation.

Pipetting errors are a plausible explanation for the fluctuations in readings, particularly intraday results. These can be improved by having someone train for longer time periods prior to the experiment (Drummer, 2007).

For cocaine and its metabolites one major source of error are the ions used for quantification. The target and qualifier ions used were not the ones suggested by the manufacturer as these were very similar. Peak 82 was quoted for all molecules of

interest, which caused problems with quantitation (Eureka, 2021). This could have caused errors as the indicated settings were not adhered to.

Morphine and its internal standard had peak 414 in common (Eureka, 2021). This could have contributed to quantification inaccuracies for the molecule. As the shared peak was present in the internal standard's spectrum this could have influenced the low readings for 6-MAM.

The methodology was performed as indicated by the manufacturer, as this is how it was implemented in the laboratory, however the obtained retention times were very close. This is also confirmed by the manufacturer data. This could have resulted in peak overlap and consequent inaccurate quantification by the apparatus. Resolution can be improved by lowering the ramp temperature, which would slow the rate of elution.

The study can be improved in various ways as follows.

The study could have been performed with a larger quantity of repeats within each run, to increase statistical significance and reduce the degree of variability. Greater efforts to carry out the tests in directly succeeding days avoid suspicions of climatic differences altering results. This approach also eliminates the variability associated with freezing and thawing of controls.

Controls could be freshly reconstituted for each run, to eliminate any variability associated with matrix changes. Further, stability tests could be performed on frozen reconstituted samples. This would eliminate any suspicion of the process of freezing and thawing being a significant factor.

It was not ideal to avoid using different kits, as to ensure robustness of the method batch to batch variability should be confirmed to not have a significant effect on results.

The significant difference between the first and second readings of all samples indicated at some inaccuracy dependant on the GC/MS apparatus itself. This could have been improved by checking the apparatus with the manufacturer to ensure it was functioning within proper parameters.

Proper analysis regarding cross reactivity could elucidate whether the presence of other similar compounds could have attributed to the inaccuracies observed in the readings. This could be done using solutions prepared from pure reagents instead of the lyophils containing various molecules of similar structure. Another approach could be deriving calibration curves for the other substances and see whether there are similar discrepancies in the obtained data for their parameters when using the lyophilised solutions. It is worth noting that in real life cases the quantification may not be as crucial to investigations, rather the presence of various similar substances all of which can contribute to a victim's death singly or combined (Konstantinova, Normann et al., 2012). This is because there are various factors influencing the quantity of each molecule and metabolite, including; gender, age, metabolic enzymes, time of death, post mortem changes and others (Konstantinova, Normann et al., 2012).

Nonetheless, quantitation is considered as a very important part of forensic analysis which helps to determine whether the substance in question was in fact the cause of death, thus its accuracy is of utmost importance (Drummer, 2007). Quantitation also influences back calculations that estimate time of death, amongst other information, thus accurate estimations are of relevance to court (Drummer, 2007).

Atest for outliers can be carried out to confirm outliers arithmetically.

4.7 Conclusion

The obtained results indicated the method developed is valid for use within the given parameters, but required further reliability testing (ENFSI). The robustness level was not satisfactory, but as previously suggested, could be further improved (SWGTOX, 2013). Some of the results indicated higher degrees of accuracy and precision are achievable via the presented method.

Further studies could further elucidate specific areas of improvement for the methodology and the apparatus to optimise the procedure, thus ensuring it is performing to the highest possible standards. Variability is to be expected by any method, but reduction of variability ensures increased confidence in obtained results and trust of the laboratory's stakeholders in providing quality forensic data (SWGTOX, 2013).

These studies are pivotal to the manufacturer prior to marketing, as they prove the functionality of the kit and are essential to be granted a licence. within toxicology laboratories, validation studies are carried out to ensure the used methodologies are reliable (SWGTOX, 2013). This is important to the forensic scientist as the results can be put to question in court (Drummer, 2007). Accuracy and precision are of particular importance to toxicology studies, as these confirm reading consistency of the method and proof the output of testing is indeed a true measurement of the parameter in question, respectively (SWGTOX, 2013). Thus, having robust data in relation to accuracy and precision provides a solid base to trust the results obtained from

unknown samples (Drummer, 2007). Forensic toxicology is the application of analytical pharmacotoxicology to the forensic field, thus improving analysis procedures in this field aids in better understanding the behaviour of xenobiotics ante and postmortem, while also using the obtained data in a practical setting to solve crime.

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Appendix 1 Results of literature review search

Title	Authors	Year	Overview
2-Nitro-6-monoacetylmorphine: potential marker for monitoring the presence of 6-monoacetylmorphine in urine adulterated with potassium nitrite	Luong, Susan; Shimmon, Ronald; Hook, James and Fu, Shanlin	2012	testing a method to detect 6-MAM in adulterated urine samples
6-Acetylmorphine Detection in Postmortem Cerebrospinal Fluid	Jenkins, Amanda J.; Lavins, Eric S.	1998	Detection of 6-MAM in cerebrospinal fluid for forensic investigation.
6-Monoacetylmorphine-antibody distribution in tissues from heroin-related death cases: An experimental study to investigate the distributive response	Maiese, Aniello; La Russa, Raffaele; David, Maria Chiara; Cantatore, Santina; Manetti, Alice Chiara; De Matteis, Alessandra; Ciallella, Costantino; Frati, Paola and Fineschi, Vittorio	2022	Detection of 6-MAM in various body tissues to observe distribution in the dead.
A Comparative Evaluation of the Instant-View 5-Panel Test Card with OnTrak TesTcup Pro 5: Comparison with Gas Chromatography-Mass Spectrometry	Moody, David E.; Fang, Wenfang B.; Andrenyak, David M.; Monti, Kim M. and Jones, Chuck	2006	testing new onsite drug detection kit via comparison with GC/MS results.
A Direct Aqueous Derivatization GSMS Method for Determining Benzoylecgonine Concentrations in Human Urine	Chericoni, Silvio; Stefanelli, Fabio; Da Valle, Ylenia and Giusiani, Mario	2015	Validation of benzoylecgonine detection method in urine via GC/MS
A high-throughput urinalysis of abused drugs based on a SPE-LC–MS/MS method coupled with an in-house developed post-analysis data treatment system	Cheng, Wing-Chi; Yau, Tsan-Sang; Wong, Ming-Kei; Chan, Lai-Ping and Mok, Vincent King-Kuen	2006	Testing high throughput urinalysis via LC/MS-MS.

A hybrid liquid chromatography–mass spectrometry strategy in a forensic laboratory for opioid, cocaine and amphetamine classes in human urine using a hybrid linear ion trap-triple quadrupole mass spectrometer	Dowling, Geraldine; Regan, Liam; Tierney, Julie and Nangle, Michael	2010	Validation of opioid, cocaine and amphetamine urinalysis method in a forensic laboratory.
A Practical Approach to Determination of Laboratory GC-MS Limits of Detection	Underwood, Paula J.; Kananen, Gerald E. and Armitage, Edwin K.	1997	Validation of urinalysis method via GC/MS.
A Practical Approach to Determine Cutoff Concentrations for Opiate Testing with Simultaneous Detection of Codeine, Morphine, and 6-Acetylmorphine in Urine	Paul, Buddha D.; Shimomura, Eric T. and Smith, Michael L.	1999	Testing limit of detection for opiates in urine.
A rapid GC–MS method for the determination of dihydrocodeine, codeine, norcodeine, morphine, normorphine and 6-MAM in urine	Meadway, C.; George, S. and Braithwaite, R.	2002	In-house testing of opiate urinalysis method via GC/MS.
Analysis of drugs of abuse in human plasma by dispersive liquid-liquid microextraction and high-performance liquid chromatography	Fernández, P.; Regenjo, M.; Bermejo, A.M.; Fernández, A. M.; Lorenzo, R.A. and Carro, A.M.	2015	In-house testing of opiate urinalysis method via HPLC.
Analysis of opiates, cocaine and metabolites in urine by high-performance liquid chromatography with diode array detection (HPLC-DAD)	Fernández, P.; Vázquez, C.; Morales, L. and Bermejo, A.M.	2005	drug detection in urine using HPLC-DAD.
Broad Spectrum Drug Identification Directly from Urine, Using Liquid Chromatography-Tandem Mass Spectrometry	Fitzgerald, Robert L.; Rivera, Jeffrey D. and Herold, David A.	1999	Method validation for LC-MS-MS urinalysis method.

Chemiluminescence detection of opium poppy (Papaver somniferum) alkaloids	Francis, Paul S.; Adcock, Jacqui L.; Costin, Jason W.; Purcell, Stuart D.; Pfeffer, Frederick M. and Barnett, Neil W.	2008	Detection of opiates via chemiluminescence for confirmation of opium use.
Chiral analysis of methorphan in opiate-overdose related deaths by using capillary electrophoresis	Bertaso, Anna; Musile, Giacomo; Gottardo, Rossella; Seri, Catia and Tagliaro, Franco	2015	Detection of methorphan in opiate overdose cases.
Chromatographic determination of drugs of abuse in vitreous humor using solid-phase extraction	Fernández, Purificación; Seoane, Santiago; Vázquez, Cristina; Tabernero, María Jesús; Carro, Antonia M. and Lorenzo, Rosa A.	2013	Detecting drugs of abuse via GC/MS in vitreous humour for forensic analysis
Cocaine and Opiates Use in Pregnancy: Detection of Drugs in Neonatal Meconium and Urine	Lopez, P.; Bermejo, A.M.; Tabernero, M.J.; Cabarcos, P.; Alvarez, I. and Fernandez, P.	2009	Testing a method to detect drug use throughout pregnancy via meconium, using urine as a comparison.
Cocaine in sudden and unexpected death: A review of 49 post-mortem cases	Pilgrim, Jennifer L.; Woodford, Noel and Drummer, Olaf H.	2013	Forensic study to explore the involvement of cocaine in sudden death.
Cocaine-related deaths: An enigma still under investigation	Bertol, Elisabetta; Trignano, Claudia; Di Milia, Maria Grazia; Di Padua, Marianna and Mari, Francesco	2008	Forensic study related to mechanisms involved in death due to cocaine use.
Comparing presumptive with direct-to-definitive drug testing in oral fluid vs. urine for a U.S. national sample of individuals misusing drugs	Magura, Stephen; Lee-Easton, Miranda; Abu-Obaid, Ruqayyah; Reed, Pete; Allgaier, Brandi; Amaratunga, Piyadarsha; Lorenz-Lemberg, Bridget; Levitas, Matthew and Achtyes, Eric D.	2023	Validation of drug testing via oral fluid samples.

Comparison between drug screening by immunoassay and ultra-high performance liquid chromatography/high-resolution time-of-flight mass spectrometry in post-mortem urine	Sundström, Mira; Pelander, Anna and Ojanperä, Ilkka	2015	Testing the efficiency of an immunoassay as opposed to LC/TFMS for forensic analysis.
Comparison of Cocaine/Crack Biomarkers Concentrations in Oral Fluid, Urine and Plasma Simultaneously Collected From Drug Users	Fiorentin, Taís Regina; Scherer, Juliana Nichterwitz; Marcelo, Marcelo Caetano Alexandre; Sousa, Tanara Rosângela Vieira; Pechansky, Flavio; Ferrão, Marco Flôres and Limberger, Renata Pereira	2018	Study to find correlation of cocaine and its metabolites concentrations in various body fluids.
Comparison of daily urine, sweat, and skin swabs among cocaine users	Kidwell, D.A.; Kidwell, J.D.; Shinohara, F.; Harper, C.; Roarty, K.; Bernadt, K.; McCaulley, R.A. and Smith, F.P.	2003	Comparative study of cocaine and related metabolites'' concentrations in various body fluids and skin swabs.
Comparison of point-of-collection screening of drugs of abuse in oral fluid with a laboratory-based urine screen	Barrett, Cindy; Good, Carl and Moore, Christine	2001	Comparative study of oral fluid point-of-collection tests to urine laboratory screening to verify reliability.
Comparison of the Microgenics CEDIA Heroin Metabolite (6-AM) and the Roche Abuscreen ONLINE Opiate Immunoassays for the Detection of Heroin Use in Forensic Urine Samples	Holler, Justin M.; Bosy, Thomas Z.; Klette, Kevin L.; Wiegand, Russel; Jemionek, John and Jacobs, Aaron	2004	Validation study for immunoassays intended for heroin use detection.
Comparison of urine results concerning co-consumption of illicit heroin and other drugs in heroin and methadone maintenance programs	Musshoff, Frank; Trafkowski, Jens; Lichtermann, Dirk and Madea, Burkhard	2010	Drug addiction study to explore the efficiency of a methadone maintenance program.
Comprehensive Analysis of Drugs of Abuse in	Ellison, Sparkle T.; Brewer, William E. and Morgan, Stephen L.	2009	Validation of a drugs of abuse urinalysis method using a novel

Urine Using Disposable Pipette Extraction			pipette extraction approach.
Concentration profiles of cocaine, pyrolytic methyl ecgonidine and thirteen metabolites in human blood and urine: determination by gas chromatography-mass spectrometry	Paul, Buddha D.; Lalani, Shairose; Bosy, Thomas; Jacobs, Aaron J. and Huestis, Marilyn A.	2005	A distribution study for cocaine and its metabolites in human body fluids via GC/MS.
Concomitant Heroin and Cocaine Use among Opioid-Dependent Patients during Methadone, Buprenorphine or Morphine Opioid Agonist Therapy	Gastberger, Salome; Baumgartner, Markus R.; Soyka, Michael; Quednow, Boris B.; Hulka, Lea M.; Herdener, Marcus; Seifritz, Erich and Mutschler, Jochen	2019	A study of drug use patterns amongst drug addicts and opioid agonist treatment.
Confirmation of recent heroin abuse: Accepting the challenge	Maas, Alexandra; Madea, Burkhard and Hess, Cornelius	2018	Heroin use detection via GC/MS.
Identifying cases of heroin toxicity where 6-acetylmorphine (6-AM) is not detected by toxicological analyses	Ellis, Ashley D.; McGwin, Gerald; Davis, Gregory G. and Dye, Daniel W.	2016	Laboratory testing to identify heroin intoxication in the absence of the main metabolite, 6-MAM.
Designing of ordered two-dimensional gold nanoparticles film for cocaine detection in human urine using surface-enhanced Raman spectroscopy	Meng, Juan; Tang, Xianghu; Zhou, Binbin; Xie, Qiwen and Yang, Liangbao	2017	Cocaine use detection via urinalysis using RAMAN spectroscopy.
Detection of 6-Acetylmorphine in Vitreous Humor and Cerebrospinal Fluid—Comparison with Urinary Analysis for Proving Heroin Administration in Opiate Fatalities	Pragst, Fritz; Spiegel, Katharina; Leuschner, Ute and Hager, Antje	1999	Verification of a method to detect heroin use via vitreous humour and and cerebrospinal fluid.
Detection of Cocaine Analytes and Opiates in Nails from Postmortem Cases	Engelhart, David A.; Jenkins, Amanda J.	2002	Forensic analysis for cocaine and opiates in nails as opposed to urine.

Detection of cocaine and its metabolites in urine using solid phase extraction-ion mobility spectrometry with alternating least squares	Lu, Yao; O'Donnell, Ryan, M. and Harrington, Peter B.	2009	Testing a novel method for the detection of cocaine and its metabolites in urine.
Detection Times and Analytical Performance of Commercial Urine Opiate Immunoassays Following Heroin Administration	Smith, Michael L.; Shimomura, Eric T.; Summers, Jacquelyn; Paul, Buddha D.; Nichols, Dan; Shippee, Ronald; Jenkins, Amanda J.; Darwin, W.D. and Cone, Edward J.	2000	Validation of opiate immunoassays.
Determination of 74 new psychoactive substances in serum using automated in-line solid-phase extraction-liquid chromatography-tandem mass spectrometry	Lehmann, Sabrina; Kieliba, Tobias; Beike, Justus; Thevis, Mario and Mercer-Chalmers-Bender, Katja	2017	Validation of a forensic simultaneous analysis method for drug detection using urine.
Determination of buprenorphine, norbuprenorphine, naloxone, and their glucuronides in urine by liquid chromatography–tandem mass spectrometry	Mariottini, Claudia; Gergov, Merja and Ojanperä, Ilkka	2021	LC/MS-MS method for medical opioid testing in urine.
Determination of cocaine and cocaethylene in plasma by solid-phase microextraction and gas chromatography–mass spectrometry	Álvarez, Iván; Bermejo, Ana María; Tabernero, María Jesús; Fernández, Purificación and López, Patricia	2007	Validation of a urinalysis method via SPE and GC/MS for the presence of cocaine and cocaethylene.
Determination of cocaine and heroin with their respective metabolites in meconium by gas chromatography-mass spectrometry	López, P.; Bermejo, A.M.; Tabernero, M.J.; Fernández, P. and Álvarez, I.	2007	Quantitation of cocaine and heroin and their metabolites in meconium via GC/MS to determine in utero exposure.
Determination of cocaine and its derivatives in hair samples by liquid phase microextraction (LPME) and gas	Pego, A.M.F.; Roveri, F.L.; Kuninari, R.Y.; Leyton, V.; Miziara, I.D. and Yonamine, M.	2017	Testing the applicability of hair as a matrix to detect cocaine use.

chromatography–mass spectrometry (GC–MS)			
Determination of cocaine and its major metabolite benzoylecgonine in several matrices obtained from deceased individuals with presumed drug consumption prior to death	Alvear, Eduardo; von Baer, Dietrich, PhD; Mardones, Claudia, and Hitschfeld, Antonieta	2014	Exploring the detectability of cocaine and its metabolites in various matrices obtained for forensic use.
Determination of cocaine and its metabolites in plasma by porous membrane-protected molecularly imprinted polymer micro-solid-phase extraction and liquid chromatography—tandem mass spectrometry	Sánchez-González, Juan; García-Carballal, Sara; Cabarcos, Pamela; Tabernero, María Jesús; Bermejo-Barrera, Pilar and Moreda-Piñeiro, Antonio	2016	Validating the efficiency of a new matrix for SPME in conjunction with LC/MS-MS to test for cocaine and its metabolites in plasma.
Determination of Drugs of Abuse in a Single Sample of Human Teeth by a Gas Chromatography-Mass Spectrometry Method	Ottaviani, Giovanni; Cameriere, Roberto; Cippitelli, Marta; Froldi, Rino; Tassoni, Giovanna; Zampi, Massimiliano and Cingolani, Mariano	2017	Exploring the use of teeth as a potential matrix for forensic drug detection.
Determination of hydroxy metabolites of cocaine from hair samples and comparison with street cocaine samples	Franz, Thomas; Scheufler, Frank; Stein, Klaus; Uhl, Michael; Dame, Torsten; Schwarz, Gerlinde; Sachs, Hans; Skopp, Gisela and Musshoff, Frank	2018	Comparing the concentration of hydroxy cocaine metabolites in addicts' hair and street samples to determine street drug composition and its correlation with users.
Determination of morphine, codeine, and thebaine concentrations from poppy seed tea using magnetic carbon nanotubes facilitated dispersive micro-solid	Li, Sun Yi; Swortwood, Madeleine J. and Yu, Jorn (Chi Chung)	2021	Observing the concentration of opium alkaloids in poppy tea users.

phase extraction and GC-MS analysis			
Determination of opiates in postmortem bone and bone marrow	Raikos, N.; Tsoukali, H. and Njau, S.N.	2001	Exploring the use of bone and bone marrow as a potential drug use detection matrix in forensics.
Determination of Opiates in Whole Blood and Vitreous Humor: A Study of the Matrix Effect and an Experimental Design to Optimize Conditions for the Enzymatic Hydrolysis of Glucuronides	Sanches, Livia Rentas; Seulin, Saskia Carolina; Leyton, Vilma; Bismara Paranhos, Beatriz, Aparecida Passos; Pasqualucci, Carlos Augusto; Muñoz, Daniel Romero; Osselton, Michael David and Yonamine, Mauricio	2012	Maximising analyte collection from whole blood and vitreous humour for drug glucuronides.
Development of a method for the determination of cocaine, cocaethylene and norcocaine in human breast milk using liquid phase microextraction and gas chromatography-mass spectrometry	Silveira, Gabriela de Oliveira; Belitsky, Íris Tikkanen; Loddi, Silvana; Rodrigues de Oliveira, Carolina Dizioli; Zucoloto, Alexandre Dias; Fruchtengarten, Ligia Veras Gimenez and Yonamine, Mauricio	2016	Testing the presence of cocaine and its metabolites in breast milk.
Direct and efficient liquid chromatographic-tandem mass spectrometric method for opiates in urine drug testing - importance of 6-acetylmorphine and reduction of analytes	Andersson, Maria; Stephanson, Nikolai; Öhman, Inger; Terzuoli, Tommy; Lindh, Jonatan D. and Beck, Olof	2014	LC/MS-MS for the detection of opiates in urine.
Distribution of opiates in femoral blood and vitreous humour in heroin/morphine-related deaths	Rees, Kelly A.; Pounder, Derrick J. and Osselton, M.D.	2013	Use of femoral blood and vitreous humour to test the distribution of opiates in heroin/morphine overdose cases.

Driving under the influence of drugs — evaluation of analytical data of drugs in oral fluid, serum and urine, and correlation with impairment symptoms	Toennes, Stefan W.; Kauert, Gerold F.; Steinmeyer, Stefan and Moeller, Manfred R.	2005	Use of oral fluid, serum, and urine to confirm drug use in drivers compared to observed symptoms of use.
Drug screening and confirmation by GC–MS: Comparison of EMIT II and Online KIMS against 10 drugs between US and England laboratories	Lu, Natalie T.; Taylor, Bruce G.	2006	Validation study for immunoassays intended for onsite drug detection compared to GC/MS.
Efficacy of DRIVEN-FLOW® M7-II, a new on-site drug screening kit in postmortem urine compared with Triage DOA	Tanaka, Toshiko; Yoshizumi, Shiomi; Kasai, Kentaro; Yoshida, Kosho and Sato, Hiroaki	2021	Comparison of results obtained from the onsite drug testing kit to the laboratory drug testing analysis.
Evaluation of a Newly Formulated Enzyme Immunoassay for the Detection of Hydrocodone and Hydromorphone in Pain Management Compliance Testing	Nascimento, Renata; Poklis, Alphonse and Wolf, Carl E.	2016	Validation of new enzyme immunoassay intended for the detection of pain medication opioids.
Evaluation of a Solid-Phase Extraction Method for Benzoylecgonine Urine Analysis in a High-Throughput Forensic Urine Drug-Testing Laboratory	Stout, Peter R.; Gehlhausen, Jay M.; Horn, Carl K. and Klette, Kevin L.	2002	Validation of SPE use in a method to detect benzoylecgonine in urine.
Evaluation of an on-site test device for the heroin metabolite 6-acetylmorphine in urine	Picht, Fabian; Beck, Olof and Böttcher, Michael	2019	Testing the efficiency of an onsite 6-MAM testing devise as opposed to laboratory methods.
Evaluation of Two Enzyme Immunoassays for the Detection of the Cocaine Metabolite Benzoylecgonine in 1,398 Urine Specimens	Carney, Sarah; Wolf, Carl E.; Tarnai-Moak, Lisa and Poklis, Alphonse	2012	Validating the use of two enzyme immunoassays to detect benzoylecgonine in urine.

Examination of Postmortem Fluids and Tissues for the Presence of Methylecgonidine, Ecgonidine, Cocaine, and Benzoylecgonine Using Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry	Shimomura, Eric T.; Hodge, Gwendolyn D. and Paul, Buddha D.	2001	Forensic study to test various post mortem fluids for the presence of cocaine and its metabolites via GC/MS, inhouse method verification.
Extraction of Benzoylecgonine from Urine Specimens with Cerex Polycrom Clin II Solid-Phase Extraction Columns and the Speedisk Pressure Processor	Kuhnle, Judith A.; Churley, Melissa; Kawasaki, Shiralen Y.; Lyons, Timothy P. and Bruins, Mark R.	2001	Testing the efficiency of two extraction devises to extract benzoylecgonine from urine.
Fatal versus non-fatal heroin “overdose”: blood morphine concentrations with fatal outcome in comparison to those of intoxicated drivers	Meissner, Christoph; Recker, Sabine; Reiter, Arthur; Friedrich, Hans Juergen and Oehmichen, Manfred	2002	Comparing blood heroin concentration to the fatality of the overdose case.
Forensic Drug Testing for Opiates. VII. Urinary Excretion Profile of Intranasal (Snorted) Heroin	Cone, Edward J.; Jufer, Rebecca; Darwin, William D. and Needleman, Saul B.	1996	Deriving an extraction profile for heroin abuse via snorting.
Formation of Benzoylecgonine Isopropyl Ester Following Solid-Phase Extraction	Juhascik, Matthew; Gleba, Jessica and Jenkins, Amanda	2012	Testing the formation of a benzoylecgonine derivative as a result of interaction with SPE matrix.
Gas chromatographic determination of cocaine and its metabolites in blood and urine from cocaine users in northwestern Spain	Fernández, P.; Buján, L.; Bermejo, A.M. and Tabernero, M.J.	2004	Testing drug use patterns in north-western Spain.
GC-FID determination of cocaine and its metabolites in human bile and vitreous humor	Fernández, P.; Aldonza, M.; Bouzas, A.; Lema, M.; Bermejo, A.M. and Tabernero, M.J.	2006	Use of bile and vitreous humour for the forensic detection of cocaine and its metabolites using GC-FID.

Gender-related differences in the pharmacokinetics of opiates	Djurendic-Brenesel, Maja; Mimica-Dukic, Neda; Pilija, Vladimir and Tasic, Milos	2010	Testing the various opiates and metabolites within body fluids of users to determine differences in metabolism amongst males and females.
Green Analytical Toxicology for the Determination of Cocaine Metabolites	de Paula Meirelles, Gabriela; Fabris, André Luis; Ferreira Dos Santos, Karina; Costa, José Luiz and Yonamine, Mauricio	2023	Designing a new eco-friendly approach to determine cocaine metabolites in urine.
Heroin use by motorists in Sweden confirmed by analysis of 6-acetylmorphine in urine	Jones, A W.	2001	Population study of heroin use in Sweden.
Heroin-Related Compounds and Metabolic Ratios in Postmortem Samples Using LC-MS-MS	Jakobsson, Gerd; Truver, Michael T.; Wrobel, Sonja A.; Gréen, Henrik and Kronstrand, Robert	2021	Deriving a metabolic profile from post-mortem samples for heroin and related compounds using LC/MS-MS.
High throughput analysis of drugs of abuse in hair by combining purposely designed sample extraction compatible with immunometric methods used for drug testing in urine	de la Torre, R; Civit, E.; Svaizer, F.; Lotti, A.; Gottardi, M. and Miozzo, M.	2010	Testing the efficiency of a high throughput method for drug detection in hair samples.
Identification of the General Unknown. Application of Mass Selective Detectors in Forensic Toxicology	Stimpfl, Thomas; Vycudilik, Walter	2000	Commentary on the use of Mass spectroscopy in forensics.
Importance of Vacutainer Selection in Forensic Toxicological Analysis of Drugs of Abuse	Toennes, Stefan W.; Kauert, Gerold F.	2001	Discussion of the relevance of choosing the right sample container when collecting samples for forensic analysis.
Instrumental and chemometric analysis of opiates via gas chromatography–vacuum	Roberson, Zackery R.; Gordon, Heather C. and Goodpaster, John V.	2020	Testing opiate presence via GC-VUV.

ultraviolet spectrophotometry (GC-VUV)			
Investigation of the identification point system adaptation in cocaine, benzoylecgonine and ecgonine methyl ester using a single quadrupole mass spectrometer	Pistos, C.; Karampela, S.; Papoutsis, I.; Athanaselis, S.; Spiliopoulou, Ch and Maravelias, C.	2009	Validation of an alternative setup to identify cocaine and its metabolites in biological samples using a single quadrupole mass spectrometer.
'Lingering' opiate deaths? Concentration of opiates in medulla and femoral blood	Naso-Kaspar, Claire; Herndon, Grant W.; Wyman, John F.; Felo, Joseph A.; Lavins, Eric S. and Gilson, Thomas P.	2013	Testing the viability of using the medulla and femoral blood to obtain and accurate opiate concentration in forensic analysis.
Metabolites of Heroin in Several Different Post-mortem Matrices	Thaulow, Cecilie Hasselø; Øiestad, Åse, Marit Leere; Rogde, Sidsel; Karinen, Ritva; Brochmann, Gerd Wenche; Andersen, Jannike Mørch; Høiseth, Gudrun; Handal, Marte; Mørland, Jørg; Arnestad, Marianne and 3 others	2018	Observing the presence of heroin metabolites in different postmortem samples
Microwave assisted extraction of drugs of abuse from human urine	Fernández, Purificación; Lago, Marta; Lorenzo, Rosa Antonia; Carro, Antonia María; Bermejo, Ana María and Tabernero, María Jesús	2007	Analysing the detection of drugs of abuse in urine when using microwave assisted extraction for the purpose.
Modified Method for Detection of Benzoylecgonine in Human Urine by GC-MS: Derivatization Using Pentafluoropropanol/Acetic Anhydride	Serafin, Michelle C.; Paulemon, Kasandra M.; Fuller, Zachary J. and Bronner, William E.	2017	Analysing the detection of benzoylecgonine in urine via GC/MS when using pentafluoropropanol and acetic anhydride to extract the molecule from the matrix.

Morphine and codeine concentrations in human urine following controlled poppy seeds administration of known opiate content	Smith, Michael L.; Nichols, Daniel C.; Underwood, Paula; Fuller, Zachary; Moser, Matthew A.; LoDico, Charles; Gorelick, David A.; Newmeyer, Matthew N.; Concheiro, Marta and Huestis, Marilyn A.	2014	Testing the quantity of morphine and codeine detected in urine after poppy seed consumption.
Morphine to codeine concentration ratio in blood and urine as a marker of illicit heroin use in forensic autopsy samples	Konstantinova, Svetlana V.; Normann, Pert.; Arnestad, Marianne; Karinen, Ritva; Christophersen, Asbjørg S. and Mørland, Jørg	2012	Observing the ratio of morphine to codeine in forensic blood samples and the relevance to detecting illicit heroin use.
Morphine, Morphine-3-Glucuronide, Morphine-6-Glucuronide, and 6-Monoacetylmorphine Determined by Means of Atmospheric Pressure Chemical Ionization-Mass Spectrometry-Liquid Chromatography in Body Fluids of Heroin Victims	Bogusz, Maciej J.; Maier, Rolf-Dieter and Driessen, Sarah	1997	Detecting various heroin metabolites and their glucuronides using LC/MS in the body fluids of heroin overdose victims.
New sorbent materials for selective extraction of cocaine and benzoylecgonine from human urine samples	Bujak, Renata; Gadzała-Kopciuch, Renata; Nowaczyk, Alicja; Raczak-Gutknecht, Joanna; Kordalewska, Marta; Struck-Lewicka, Wiktoria; Waszczuk-Jankowska, Ma; Tomczak, Ewa; Kaliszan, Michał; Buszewski, Bogusław and	2016	Testing extraction efficiency of various sorbent materials for cocaine and benzoylecgonine in urine.
Novel Receptor-Binding-Based Assay for the Detection of Opioids in Human Urine Samples	Bergerhoff, Maja; Moosmann, Bjoern	2023	Testing the efficiency of a new immunoassay for opiates.
On-site testing of illicit drugs: the use of the	Biermann, T.; Schwarze, B.; Zedler, B. and Betz, P.	2004	Validation of the onsite “Toxiquick ®"

drug-testing device “Toxiquick ®"			devise for drug detection.
Overview of cocaine identification by vibrational spectroscopy and chemometrics	John, Danielle Kochenborger; dos Santos Souza, Klester and Ferrão, Marco Flôres	2023	Testing the applicability of vibrational spectroscopy and chemometrics to identify cocaine.
Paradoxical Results in Urine Drug Testing for 6-Acetylmorphine and Total Opiates: Implications for Best Analytical Strategy	Beck, Olof; Bottcher, Michael	2006	Discussion of contradicting results related to 6-MAM and total opiate concentration and the best approach to analysis.
Performance of immunoassays in screening for opiates, cannabinoids and amphetamines in post-mortem blood	Hino, Yukiko; Ojanperä, Ilkka; Rasanen, Ilpo and Vuori, Erkki	2003	Testing the performance of immunoassays to detect opiates, cannabinoids and amphetamines in forensic blood testing.
Pharmacokinetic interactions between ethanol and heroin: A study on post-mortem cases	Thaulow, C.H.; Høiseth, G; Andersen, J.M.; Handal, M. and Mørland, J	2014	Analysing post-mortem body fluids for and chemical interaction between heroin and ethanol in victims of overdose.
Porous membrane-protected molecularly imprinted polymer micro-solid-phase extraction for analysis of urinary cocaine and its metabolites using liquid chromatography – Tandem mass spectrometry	Sánchez-González, Juan; Tabernero, María Jesús; Bermejo, Ana María; Bermejo-Barrera, Pilar and Moreda-Piñeiro, Antonio	2015	Testing the applicability of a new material to SPE in the analysis of cocaine and its metabolites via LC/MS-MS.
Postmortem Fluid Concentrations of Heroin Biomarkers and Their Metabolites	Al-Asmari, Ahmed I.	2020	Deriving concentration profile of heroin and its metabolites in post-mortem fluids.

Postmortem redistribution of cocaine and its metabolites, benzoylecgonine and ecgonine methyl ester in humans: Important variables that might be influencing the central blood / peripheral blood ratio	Emaus, Robin-Alissa; Borra, Lennaert Christiaan Pieter; van der Hulst, Rogier; Kloos, Dick-Paul; Rijken, Dingeman Johannes; Elsinga, Philip Hein; Boersma, Hendrikus Hessel; Bosman, Ingrid Jolanda and Touw, Daniel Johannes	2023	Analysing post mortem redistribution of cocaine and its metabolites, and its impact on the obtained concentrations within central and peripheral blood samples.
Prediction of Impairment from Urine Benzoylecgonine Concentrations	Moore, Karla A.; Levine, Barry and Fowler, David R.	2003	Testing urine benzoylecgonine concentration and comparing this to the level of impairment observed in patients.
Quantification of Five Compounds with Heterogeneous Physicochemical Properties (Morphine, 6-Monoacetylmorphine, Cyamemazine, Meprobamate and Caffeine) in 11 Fluids and Tissues, Using Automated Solid-Phase Extraction and Gas Chromatography–Tandem Mass Spectrometry	Bévalot, Fabien; Bottinelli, Charline; Cartiser, Nathalie; Fanton, Laurent and Guitton, Jérôme	2014	Method validation for a new procedure to detect 5 different molecules in several body fluids simultaneously using SPE and GC/MS-MS.
Quantitative analysis of cocaine and its metabolites in whole blood and urine by high-performance liquid chromatography coupled with tandem mass spectrometry	Johansen, Sys Stybe; Bhatia, Helle Merete	2007	Using LC/MS-MS to quantify cocaine and its metabolites in blood and urine.
Quantitative determination of amphetamines, cocaine, and opiates in human hair by gas	Skender, L.; Karačić, V; Brčić, I and Bagarić, A	2002	Using GC/MS to detect opiates, cocaine and amphetamines in hair samples.

chromatography/mass spectrometry			
Rapid Analysis of Benzoylecgonine in Urine by Fast Gas Chromatography-Mass Spectrometry	Romberg, Robert W.; Jamerson, Matthew H. and Klette, Kevin L.	2006	Testing the use of a fast GC/MS method to detect benzoylecgonine in urine.
Rapid and semi-quantitative presumptive tests for opiate drugs	Choodum, Aree; Nic Daeid, Niamh	2011	Testing the reliability of semiquantitative kits for opiate use.
Rapid screening of drugs of abuse in human urine by high-performance liquid chromatography coupled with high resolution and high mass accuracy hybrid linear ion trap-Orbitrap mass spectrometry	Li, Xiaowen; Shen, Baohua; Jiang, Zheng; Huang,Yi and Zhuo, Xianyi	2013	Testing HPLC/MS use for rapid drugs of abuse screening.
Rapid simultaneous determination of ephedrines, amphetamines, cocaine, cocaine metabolites, and opiates in human urine by GC–MS	Saito, Takeshi; Mase, Hiroyasu; Takeichi, Sanae and Inokuchi, Sadaki	2007	Testing for ephedrines, amphetamines, cocaine and its metabolites, and opiates in urine using GC/MS.
Requirements for bioanalytical procedures in postmortem toxicology	Drummer, Olaf H.	2007	Overview of the methods and requirements for analysis in post-mortem toxicology.
Response to Ruan and colleagues concerning 'The toxicology of heroin-related death: estimating survival times'	Darke, Shane; Duflou, Johan	2016	Discussing the time of death of heroin overdose victims.
Results of hair analyses for drugs of abuse and comparison with self-reports and urine tests	Musshoff, F.; Driever, F.; Lachenmeier, K.; Lachenmeier, D.W.; Banger, M. and Madea, B.	2006	Comparing the results of hair drug concentration, urine drug concentration and self-reports.
Sensitive Method For Detection Of Cocaine And Associated Analytes By Liquid Chromatography-Tandem Mass	LANGMAN, Loralie J.; BJERGUM, Matthew W.; WILLIAMSON, Christopher L. and CROW, Frank W.	2009	A verified method to detect cocaine and its metabolites in urine using LC/MS-MS.

Spectrometry In Urine: Society of Forensic Toxicologist, Inc			
Simultaneous determination of 75 abuse drugs including amphetamines, benzodiazepines, cocaine, opioids, piperazines, zolpidem and metabolites in human hair samples using liquid chromatography–tandem mass spectrometry	Shin, Yongho; Kong, Tae Yeon; Cheong, Jae Cheol; Kim, Jin Young; Lee, Jae Il and Lee, Hye Suk	2019	Using LC/MS-MS to determine the presence of various drugs simultaneously in human hair samples.
Simultaneous determination of cocaine, ecgonine methyl ester, benzoylecgonine, cocaethylene and norcocaine in dried blood spots by ultra-performance liquid chromatography coupled to tandem mass spectrometry	de Lima Feltraco Lizot, Lilian; da Silva, Anne, Caroline Cezimbra; Bastiani, Marcos Frank; Hahn, Roberta Zilles; Bulcão, Rachel; Perassolo, Magda Susana; Antunes, Marina Venzon and Linden, Rafael	2019	Testing the use of LC/MS-MS to detect cocaine and its metabolites in dried blood spots.
Simultaneous determination of morphine, codeine and 6-acetyl morphine in human urine and blood samples using direct aqueous derivatisation: Validation and application to real cases	Chericoni, S.; Stefanelli, F.; Iannella, V. and Giusiani, M.	2014	Validating a method to detect opiates in urine and blood via aqueous derivatisation.
Simultaneous Determination of Xylazine, Free Morphine, Codeine, 6-Acetylmorphine, Cocaine and Benzoylecgonine in Postmortem Blood by UPLC-MS-MS	Ruiz-Colón, Kazandra; Martínez, María, A.; Silva-Torres, Luz; Chavez-Arias, Carlos; Meléndez-Negrón, Margarita; Conte-Miller, Mar and Bloom-Oquendo, Joseph	2012	Validating a UPLC-MS-MS method to detect simultaneously various drugs in post-mortem blood, including opiates cocaine ad its metabolite.

Simultaneous Quantitation of Methamphetamine, Ketamine, Opiates and their Metabolites in Urine by SPE and LC–MS-MS	Yang, Chu-An; Liu, Hsiu-Chuan; Lin, Dong-Liang; Liu, Ray H.; Hsieh, You-Zung and Wu, Shu-Pao	2017	Using LC/MS-MS to detect simultaneously opiates, methamphetamine and ketamine in urine.
Simultaneous Quantitation of Opioids in Blood by GC-EI-MS Analysis Following Deproteination, Detautomerization of Keto Analytes, Solid-Phase Extraction, and Trimethylsilyl Derivatization	Ropero-Miller, Jeri; Lambing, Matthew K. and Winecker, Ruth E.	2002	Using blood to quantify opioids via GC-EI-MS.
Solid-phase microextraction for the detection of codeine, morphine and 6-monoacetylmorphine in human hair by gas chromatography–mass spectrometry	Moller, M.; Aleksa, K.; Walasek, P.; Karaskov, T. and Koren, G.	2010	Validating a method for opiate testing in hair samples using GC/MS.
Soluble manganese (IV) as a chemiluminescence reagent for the determination of opiate alkaloids, indoles and analytes of forensic interest	Brown, Allyson J.; Lenehan, Claire E.; Francis, Paul S.; Dunstan, David E. and Barnett, Neil W.	2007	Using chemiluminescence to determine opiates, indoles and other molecules of forensic interest.
Some Advances in Fourier Transform Infrared Transflection Analysis and Potential Applications in Forensic Chemistry	Kocak, A.; Lucania, J.P. and Berets, S.L.	2009	Analysing the applications of Fourier Transform in forensic chemistry.
Stability studies in biological fluids during post-analysis custody. Opiate compounds derived from heroin consumption	Huertas, T.; Jurado, C.; Salguero, M.; Soriano, T. and Gamero, J.	2019	Testing the stability of opiate metabolites of heroin in forensic samples during custody.
Stimulant use in suicides: A systematic review	Mantinieks, Dylan; Schumann, Jennifer; Drummer, Olaf H.; Woodford, Noel W.	2022	A systematic review of the involvement of stimulants in suicide cases.

	and Gerostamoulos, Dimitri		
Suspicion of driving under the influence of alcohol or drugs: Cross sectional analysis of drug prevalence in the context of the Swiss legislation	Maurer, Jonathan; Vergalito, Emeline; Prior, Anne-Flore; Donzé, Nicolas; Thomas, Aurélien and Augsburger, Marc	2021	A population study related to alcohol and drug use by Swiss drivers.
The Application of Supercritical Fluid Extraction to Cocaine and Its Metabolites in Blood and Urine	Allen, Desiree L.; Oliver, John S.	2000	Testing the use of supercritical fluid extraction to test blood and urine for cocaine and its metabolites.
The clavicle bone as an alternative matrix in forensic toxicological analysis	Vardakou, Ioanna; Athanaselis, Sotiris; Pistos, Constantinos; Papadodima, Stauroula; Spiliopoulou, Chara and Moraitis, Konstantinos	2014	Testing the viability of the clavicle bone as a matrix for forensic toxicology.
The detection of acetylcodeine and 6-acetylmorphine in opiate positive urines	O'Neal, Carol L.; Poklis, Alphonse	1998	Testing urine samples via GC/MS for acetyl codeine and 6-MAM.
Thin Layer Chromatography coupled to Paper Spray Ionization Mass Spectrometry for Cocaine and its Adulterants Analysis	De Carvalho, Thays, C.; Tosato, Flavia; Souza, Lindamara M.; Santos, Heloa; Merlo, Bianca B.; Ortiz, Rafael S.; Rodrigues, Rayza R.T.; Filgueiras, Paulo R.; França, Hildegardo, S.; Augusti, Rodinei and 2 others	2016	Testing a method to detect cocaine and its adulterants via TLC and spray ionisation mass spectrometry.
Urinalysis of Body Packers in Japan	Nihira, Makoto; Hayashida, Makiko; Ohno, Youkichi; Inuzuka, Sho; Yokota, Hiroyuki and Yamamoto, Yasuhiro	1998	Testing the urine drug concentrations of body packers in Japan via GC/MS.
Urinary Excretion of Ecgonine and Five Other Cocaine Metabolites Following Controlled Oral, Intravenous,	Smith, Michael L.; Shimomura, Eric; Paul, Buddha D.; Cone, Edward J.; Darwin,	2010	Analysis of excretion patterns of cocaine and its metabolites via urinalysis.

Intranasal, and Smoked Administration of Cocaine	W.D. and Huestis, Marilyn A.		
Usefulness of Roadside Urine Drug Screening in Drivers Suspected of Driving Under the Influence of Drugs (DUID)	Raes, Elke; Verstraete, Alain G.	2005	Discussing the relevance of urine roadside drug tests to detect drivers under the influence.
Validation of a Gas Chromatography-Ion Trap-Tandem Mass Spectrometry Assay for the Simultaneous Quantification of Cocaine, Benzoylecgonine, Cocaethylene, Morphine, Codeine, and 6-Acetylmorphine in Aqueous Solution, Blood, and Skeletal Muscle Tissue	Rees, Kelly A.; McLaughlin, Poppy A. and Osselton, M.D.	2012	Validation of a new inhouse methodology to detect various drugs of abuse in aqueous solution, blood and skeletal muscle tissue and quantify these substances simultaneously.
Validation of a Procedure for the Gas Chromatography-Mass Spectrometry Analysis of Cocaine and Metabolites in Pericardial Fluid	Contreras, María Teresa; González, Marisa; González, Susana; Ventura, Rosa; Valverde, Juan Luis; Hernández, Antonio F.; Pla, Antonio; Vingut, Albert; Segura, Jordi and de la Torre, Rafael	2007	Validation of a method to detect cocaine and its metabolites in pericardial fluid using GC/MS.
Validation of an Extraction and Gas Chromatography-Mass Spectrometry Quantification Method for Cocaine, Methadone, and Morphine in Postmortem Adipose Tissue	Colucci, A.P.; Aventaggiato, L.; Centrone, M. and Gagliano-Candela, R	2010	Validating the use of adipose tissue as a matrix to quantify cocaine, methadone and morphine via GC/MS.
What a validation strategy means for the quantitation of cocaine and heroin?	Dujourdy, Laurence; Charvoz, Céline; Dalmasso, Marion and Dufour, Anne-Béatrice	2015	Discussing the relevance of validation of cocaine and heroin quantitation methods.
Automated solid-phase extraction of opiates from urine (II).	M K Tay, T K Lee, W Y Chui	1993	Testing a new automated extraction method against the

Establishing criteria for the detection of opiate abuse.			established liquid-liquid extraction method.
Automated solid-phase extraction of opiates from urine (I).	M K Tay, T K Lee, W Y Chui	1993	Testing an C18 solid phase extraction column for opiate extraction.
Identification of drugs and other toxic compounds from their ultraviolet spectra. Part III: Ultraviolet absorption properties of 22 structural groups.	T J Siek, R J Osiewicz, R J Bath	1976	Derivation of UV spectra for various compounds.
Forensic drug testing for opiates. V. Urine testing for heroin, morphine, and codeine with commercial opiate immunoassays.	E J Cone, S Dickerson, B D Paul, J M Mitchell	1993	Testing different immunoassays for opiate extraction.
[Comparison of morphine and codeine concentration in urines of heroin abusers and codeine users].	Jun Bu, Chang-Shu Zhan	2012	Comparing the morphine and codeine concentrations in urine of codeine and heroin users.
The detection of 6-monoacetylmorphine in urine, serum and hair by GC/MS and RIA.	M R Moeller, C Mueller	1995	Validation of new radioimmunoassay for opiate detection.
Gas chromatographic/mass spectrometric analysis of morphine and codeine in human urine of poppy seed eaters.	H N elSohly, D F Stanford, A B Jones, M A elSohly, H Snyder, C Pedersen	1988	GC/MS analysis of morphine and codeine levels in urine of poppy seed consumers.
Forensic drug testing for opiates: I. Detection of 6-acetylmorphine in urine as an indicator of recent heroin exposure; drug and assay considerations and detection times.	E J Cone, P Welch, J M Mitchell, B D Paul	1991	Metabolism and elimination study for 6-MAM in urine.
Simultaneous determination of 6-monoacetylmorphine, morphine and codeine in urine using high-performance liquid	J Gerostamoulos, K Crump, I M McIntyre, O H Drummer	1993	Validation of method to detect opiates in urine via HPLC-UV Electrochemical detection.

chromatography with combined ultraviolet and electrochemical detection.			
A quantitative densitometric determination of heroin and cocaine samples by high-performance thin-layer chromatography.	E Della Casa, G Martone	1986	Testing a quantitative heroin and cocaine detection method using TLC.
Detection of opiates in urine by means of thin-layer immunoassay.	M E Fredenburg, N T Lappas	1982	Method validation for opiate detection in urine using thin layer immunoassay.
A multivariate analysis of the infrared spectra of drugs of abuse.	W W Moss, F T Posey, P C Peterson	1980	Analysing the infrared spectra of 15 commonly abused drugs.
[Simultaneous determination of opioid compounds in human urine by UPLC-MS/MS].	Chang-Shu Zhan, Bao-Hua Shen, Xian-Yi Zhuo	2010	Detecting various opioids and their glucuronide derivatives in urine using UPLC-MS/MS.
Distinction among eight opiate drugs in urine by gas chromatography-mass spectrometry.	W Nowatzke, J Zeng, A Saunders, A Bohrer, J Koenig, J Turk	1999	Formulating a method to differentiate amongst 8 opiates using GC/MS.
[Analysis of 37 drugs in whole blood by HPLC after solid phase extraction].	Chen Liang, Yu-Rong Zhang, Qi-Yun Jin, You-Mei Guo	2006	Validating a method to determine 37 different drugs in blood using HPLC and solid phase extraction.
Comparison of phenyl-type columns in the development of a fast liquid chromatographic system for eighteen opiates commonly found in forensic toxicology.	R Dams, W E Lambert, K M Clauwaert, A P De Leenheer	2000	Determining the efficiency of various extraction columns for the extraction of 18 different opiates.
Simultaneous analysis of codeine, morphine, and heroin after B-glucuronidase hydrolysis.	M Zezulak, J Snyder, S B Needleman	1993	Testing the efficiency of B-glucuronidase enzyme digestion in samples.
Comparison of results for quantitative determination of morphine by	V R Spiehler, D Reed, R H Cravey, W P Wilcox, R F Shaw, S Holland	1975	Comparative analysis of various immunoassays.

radioimmunoassay, enzyme immunoassay, and spectrofluorometry.			
Considerations in the interpretation of urine analyses in suspected opiate intoxications.	B Levine, J E Smialek	1998	Validation of the Roch Abuscreen immunoassay.
Solid phase extraction of morphine and its metabolites from postmortem blood.	J Gerostamoulos, O H Drummer	1998	Determining the efficacy of a method using HPLC UCD and UV to detect and quantify opiate concentrations in urine.
Liquid-solid extraction of lyophilized biological material for forensic analysis. I. Application to urine samples for detection of drugs of abuse.	J R Broich, D B Hoffman, S J Goldner, S Andryauskas, C J Umberger	1971	Testing a new extraction method for the detection of drugs of abuse in urine.
A review of some GLD-FID derivatization techniques found useful in forensic toxicology.	G Cimbura, J Kofoed	1974	Discussing various methods of GLD-FID derivatisation techniques used in forensics.
Forensic drug testing for opiates. IV. Analytical sensitivity, specificity, and accuracy of commercial urine opiate immunoassays.	E J Cone, S Dickerson, B D Paul, J M Mitchell	1992	Validation of various commercial immunoassays for the detection of opiates.
A solid phase extraction technique for the isolation and identification of opiates in urine.	W Huang, W Andollo, W L Hearn	1992	Testing a new SPE method in conjunction with an established GC/MS procedure for identifying opiates in urine.
Improved GC/MS analysis of opiates with use of oxime-TMS derivatives.	M Cremese, A H Wu, G Cassella, E O'Connor, K Rymut, D W Hill	1998	Testing a GC/MS opiate detection method using a silylating agent.
[Use of thin layer chromatographic analysis in forensic medical analysis of urine for opiates].	N A Gorbacheva, A M Orlova	2003	Testing various solvents to separate and identify opiates using TLC.

Determination of morphine and its 3- and 6-glucuronides, codeine, codeine-glucuronide and 6-monoacetylmorphine in body fluids by liquid chromatography atmospheric pressure chemical ionization mass spectrometry.	M J Bogusz, R D Maier, M Erkens, S Driessen	1997	Novel assay for opiate glucuronides in body fluids using LC-APCI-MS.
Clinical urinalysis of drugs and alcohol in instances of suspected surreptitious administration ("spiked drinks").	Simon P Elliott, Victoria Burgess	2005	Comparing the drug concentrations in urine obtained from various methods of detection for rape drug cases.
Liquid chromatography-mass spectrometry in forensic and clinical toxicology.	H H Maurer	1998	Review of the uses of LC-MS in forensic and clinical toxicology.
Simultaneous quantification of opiates and effect of pigmentation on its deposition in hair.	Sooyeun Lee, Eunyoung Han, Eunmi Kim, Hwakyung Choi, Heesun Chung, Seung Min Oh, Young Mi Yun, Seok Hun Jwa, Kyu Hyuck Chung	2010	Quantifying opiates in hair samples using GC/MS and observing the effects of hair dye on drug detection.
The reliability of immunoassay for determining the presence of opiates in the forensic setting.	D Kimberley Molina, Vincent J Dimaio	2005	Testing the reliability of immunoassays to detect opiates in a forensic setting.
Capillary electrophoresis for the investigation of illicit drugs in hair: determination of cocaine and morphine.	F Tagliaro, C Poiesi, R Aiello, R Dorizzi, S Ghielmi, M Marigo	1993	Using capillary electrophoresis to detect opiates in hair samples.
Significance of Morphine Concentration in Bile, Liver, and Blood: Analysis of 52 Cases of Heroin Overdoses.	Isabella Mercurio, Gianluigi Ceraso, Paola Melai, Alessio Gili, Gianmarco Troiano, Fausto Agostinelli, Massimo Lancia, Mauro Bacci	2019	Deriving heroin concentrations of bile, liver and blood using GC/MS.

[Assessment of the intake of opiates (heroin, morphine, codeine and ethylmorphine) by the analysis of intermediate metabolites in the urine: which are the criteria to adopt?].	L Rivier, C Staub, C Giroud	1991	Discussing the various methods to identify heroin users and distinguish these from patients under opiate medication.
Simultaneous analysis of thebaine, 6-MAM and six abused opiates in postmortem fluids and tissues using Zymark automated solid-phase extraction and gas chromatography-mass spectrometry.	R J Lewis, R D Johnson, R A Hattrup	2005	Testing a method to detect various opiates simultaneously in post mortem samples using SPE and GC/MS.
Trends in occurrence of drugs of abuse in blood and urine of arrested drivers and drug traffickers in the border region of Aachen.	K H Schiwy-Bochat, M Bogusz, J A Vega, H Althoff	1995	Population study related to drug use and trafficking.
Enzyme-linked immunosorbent assay and latex agglutination inhibition reaction test for cocaine and benzoylecgonine in urine.	K Aoki, Y Shikama, T Yoshida, Y Kuroiwa	1996	Testing the efficiency of a new inhouse method for drug detection.
Rapid confirmation of enzyme multiplied immunoassay technique (EMIT) cocaine positive urine samples by capillary gas-liquid chromatography/nitrogen phosphorus detection (GLC/NPD).	K Verebey, A DePace	1989	Validation of new inhouse method for urine samples drug testing.
Determination of illicit drugs and their metabolites in human urine by liquid chromatography tandem mass spectrometry including relative ion intensity criterion.	Marta Concheiro, Ana De Castro, Oscar Quintela, Angelines Cruz, Manuel López-Rivadulla	2007	Validation of new LC/MS-MS method to detect drugs in urine.

Confirmatory tests for drugs in the workplace by gas chromatography-mass spectrometry.	B A Goldberger, E J Cone	1994	Review of GC/MS methods of drug detection in urine for common drugs of abuse used in the context of workplace testing.
Incidence of cocaine metabolites in urine specimens from medical examiners' cases.	S C Harris, H E Hamilton, J E Wallace	1979	Testing the applicability of isobutane as a solvent with a mass spectrometer.
Validity testing of the TDx Cocaine Metabolite Assay with human specimens obtained after intravenous cocaine administration.	E J Cone, S L Menchen, J Mitchell	1988	Comparing results obtained from immunoassays to GC/MS results for urine drug concentrations.
Validity testing of commercial urine cocaine metabolite assays: II. Sensitivity, specificity, accuracy, and confirmation by gas chromatography/mass spectrometry.	E J Cone, J Mitchell	1989	Validation of commercial immunoassays by GC/MS confirmation.
A comparison of meconium, maternal urine and neonatal urine for detection of maternal drug use during pregnancy.	W E Wingert, M S Feldman, M H Kim, L Noble, I Hand, J J Yoon	1994	Testing drug concentrations in urine and meconium to monitor drug use during pregnancy.
Cocaine in hair, saliva, skin swabs, and urine of cocaine users' children.	F P Smith, D A Kidwell	1996	Testing cocaine concentration in various matrices to detect cocaine use in mothers and any passive drug absorption by other house inhabitants.
Application of the multiple drugs immunoassay test for rapid detection of drug abuse in postmortem urine.	Smith Srisont, Thamrong Chirachariyavej, A V M Vichan Peonim	2010	Verifying the efficiency of an immunoassay to detect and distinguish various drugs, including opiates, in post mortem urine

			samples, compared to a laboratory approach.
Aqueous phase hexylchloroformate derivatization and solid phase microextraction: determination of benzoylecgonine in urine by gas chromatography-quadrupole ion trap mass spectrometry.	B J Hall, A R Parikh, J S Brodbelt	1999	Comparing the efficiency of hexylchloroformate and SPME for cleaning benzoylecgonine prior to GC/MS.
Screening for cocaine metabolite fails to detect an intoxication.	Jacquelyn E Baker 1, Amanda J Jenkins	2008	Case reports of false negative results for cocaine and benzoylecgonine.
Gas-liquid chromatographic determination of morphine, heroin, and cocaine.	M J Prager, S M Harrington, T F Governo	1979	Validation of a gas liquid chromatography method to detect morphine, heroin and cocaine.
GC/MS quantitation of benzoylecgonine following liquid-liquid extraction of urine.	J Gerlits	1993	Recovery study for benzoylecgonine using liquid-liquid extraction prior to GC/MS.
[Analysis of heroin samples with capillary gas chromatography. Comparison of glass capillary column and packed column].	M Gloger, H Neumann	1983	Comparative study of gas capillary columns and packed columns for gas chromatography of opiates and cocaine.
Improved detection and identification of basic drugs extracted from tissue using TLC.	L Galante, J Bonventre, H Salvione, M L Bastos	1982	Testing a separation technique applicable to mixed drug samples using TLC.
[The use of a method of solid-phase extraction for sample preparation in the identification of unknown substances by IR spectroscopy].	A V Beliaev, V I Sorokin, O S Orlova, T B Kimstach	1997	Testing the efficiency of SPE columns for a method using IR spectroscopy to detect various drugs of abuse.
A comparison of ONTRAK TESTCUP, abuscreen ONTRAK, abuscreen ONLINE, and GC/MS urinalysis test results.	D J Crouch, M L Cheever, D M Andrenyak, D J Kuntz, D L Loughmiller	1998	Validation of the use of various immunoassays, efficiency compared to GC/MS test results.

Identification of unique cocaine metabolites and smoking by-products in postmortem blood and urine specimens.	A J Jenkins, B A Goldberger	1997	Testing for various cocaine metabolites and combustion by products in postmortem blood and urine samples.
Prevalence of drugs of abuse in urine of drivers involved in road accidents in France: a collaborative study.	P Marquet, P A Delpla, S Kerguelen, J Bremond, F Facy, M Garnier, B Guery, M Lhermitte, D Mathé, A L Pelissier, C Renaudeau, P Vest, J P Seguela	1998	Population study of drug use amongst road accident victims in France.
Solid-phase extraction of drugs from biological tissues--a review.	J Scheurer, C M Moore	1992	Discussing the reasons behind slow implementation of SPE clean up methods in forensic laboratories.
Hair and urine analysis: relative distribution of drugs and their metabolites.	A M Bermejo Barrera, S Strano Rossi	1995	Comparative study of blood and hair drug concentrations in multiple drug users, testing for heroin, cocaine and their derivatives.
A high-performance thin-layer chromatographic technique to screen cocaine in urine samples.	Mauricio Yonamine, Mônica Cortez Sampaio	2006	Testing the efficiency of a high-performance TLC method for cocaine detection in urine.
Validity testing of commercial urine cocaine metabolite assays: IV. Evaluation of the EMIT d.a.u. cocaine metabolite assay in a quantitative mode for detection of cocaine metabolite.	E J Cone, D Yousefnejad, S L Dickerson	1990	Validation study for a commercial immunoassay for benzoylecgonine, compared to GC/MS.
Identification of anhydroecgonine ethyl ester in the urine of a drug overdose victim.	Alan L Myers, Heather E Williams, James C Kraner, Patrick S Callery	2005	A urine sample was tested for anhydroecgonine methyl ester using both GC/MS and LC/MS to test if the compound's presence

			is legitimate or a method artefact.
Relationships between concentrations of cocaine and its hydrolysates in peripheral blood, heart blood, vitreous humor and urine.	Wayne C Duer, Daniel J Spitz, Shallyn McFarland	2006	Pharmacokinetic study of cocaine and its metabolites.
Quantitation of cocaine, benzoylecgonine, ecgonine methyl ester, and cocaethylene in urine and blood using gas chromatography-mass spectrometry (GC-MS).	Steven W Fleming, Amitava Dasgupta, Uttam Garg	2010	Method validation of an inhouse procedure to detect cocaine and its metabolites in urine and blood via GC/MS.
Determination of cocaine and benzoylecgonine by derivatization with iodomethane-D3 or PFPA/HFIP in human blood and urine using GC/MS (EI or PCI mode).	R E Aderjan, G Schmitt, M Wu, C Meyer	1993	Testing a new method to detect cocaine and benzoylecgonine in blood and urine via GC/MS.
The determination of cocaine and its major metabolite, benzoylecgonine, in postmortem fluids and tissues by computerized gas chromatography/mass spectrometry.	E C Griesemer, Y Liu, R D Budd, L Raftogianis, T T Noguchi	1983	Testing a computerised GC/MS method to detect cocaine and benzoylecgonine.
GC/MS analysis of m-hydroxybenzoylecgonine in urine. Forensic implication in cocaine use.	M A elSohly, W J Kopycki, S Feng, T P Murphy, B J Lukey	1998	Testing a method to quantify m-hydroxybenzoylecgonine in urine samples positive for cocaine and its value as a cocaine use marker.
Direct analysis for cocaine in urine by high-performance liquid chromatography (HPLC) using a column-switching technique.	A K Larsen Jr, I R Tebbett	1992	Testing a new HPLC method to detect cocaine in urine samples.
[Clinical and analytical toxicology of opiate,	Catherine Feliu, Aurélie Fouley, Hervé	2015	Comparing a GC/MS and LC/MS-MS method to detect

cocaine and amphetamine].	Millart, Claire Gozalo, Hélène Marty, Zoubir Djerada		drugs of abuse in urine.
Determination of cocaine and benzoylecgonine in human amniotic fluid using high flow solid-phase extraction columns and HPLC.	C Moore, S Browne, I Tebbett, A Negrusz, W Meyer, L Jain	1992	Validating SPE to extract cocaine and benzoylecgonine from amniotic fluid for HPLC.
Postmortem stability of cocaine and cocaethylene in blood and tissues of humans and rabbits.	F Moriya, Y Hashimoto	1996	Stability study of cocaine and its metabolites in postmortem samples.
The interpretation of cocaine and benzoylecgonine concentrations in postmortem cases.	A J Jenkins, B Levine, J Titus, J E Smialek	1999	Using cocaine concentration to determine cause of death.
Simultaneous determination of opiates, cocaine and major metabolites of cocaine in human hair by gas chromatography/mass spectrometry (GC/MS).	P Kintz, P Mangin	1995	Determining drug levels in hair samples via GC/MS after liquid-liquid extraction and silylation.
Retrospective monitoring of long-term recreational and dependent cocaine use in toenail clippings/scrapings as an alternative to hair.	Milena M Madry, Andrea E Steuer, Matthias Vonmoos, Boris B Quednow, Markus R Baumgartner, Thomas Kraemer	2014	Comparing drug concentration in nail clippings and hair to see if nails are a good alternative to hair.
Forensic immunochemistry.	K Aoki, T Yoshida, Y Kuroiwa	1996	Discussing the advantages and disadvantages of immunoassays in forensics.
Rapid detection of cocaine, benzoylecgonine and methylecgonine in fingerprints using surface mass spectrometry.	Melanie J Bailey, Robert Bradshaw, Simona Francese, Tara L Salter, Catia Costa, Mahado Ismail, Roger P Webb, Ingrid Bosman, Kim Wolff, Marcel de Puit	2015	Novel approach to detect cocaine and its metabolites in fingerprints.

Criteria for the interpretation of cocaine levels in human biological samples and their relation to the cause of death.	Boyd G Stephens, Jeffrey M Jentzen, Steven Karch, Deborah C Mash, Charles V Wetli	2004	Discussing the involvement of cocaine intoxication and cocaine levels in human samples I death cases.
Detection of drugs of forensic importance in postmortem bone.	Kelly K McGrath, Amanda J Jenkins	2009	Testing the applicability of bone as a drug test sample in forensic examinations.

Automated solid-phase extraction of opiates from urine (II). Establishing criteria for the detection of opiate abuse.	M K Tay, T K Lee, W Y Chui	1993
Automated solid-phase extraction of opiates from urine (I).	M K Tay, T K Lee, W Y Chui	1993
Identification of drugs and other toxic compounds from their ultraviolet spectra. Part III: Ultraviolet absorption properties of 22 structural groups.	T J Siek, R J Osiewicz, R J Bath	1976
Forensic drug testing for opiates. V. Urine testing for heroin, morphine, and codeine with commercial opiate immunoassays.	E J Cone, S Dickerson, B D Paul, J M Mitchell	1993
[Comparison of morphine and codeine concentration in urines of heroin abusers and codeine users].	Jun Bu, Chang-Shu Zhan	2012
The detection of 6-monoacetylmorphine in urine, serum and hair by GC/MS and RIA.	M R Moeller, C Mueller	1995
Gas chromatographic/mass spectrometric analysis of morphine and codeine in human urine of poppy seed eaters.	H N elSohly, D F Stanford, A B Jones, M A elSohly, H Snyder, C Pedersen	1988
Forensic drug testing for opiates: I. Detection of 6-acetylmorphine in urine as an indicator of recent heroin exposure; drug and assay considerations and detection times.	E J Cone, P Welch, J M Mitchell, B D Paul	1991

Simultaneous determination of 6-monoacetylmorphine, morphine and codeine in urine using high-performance liquid chromatography with combined ultraviolet and electrochemical detection.	J Gerostamoulos, K Crump, I M McIntyre, O H Drummer	1993
A quantitative densitometric determination of heroin and cocaine samples by high-performance thin-layer chromatography.	E Della Casa, G Martone	1986
Detection of opiates in urine by means of thin-layer immunoassay.	M E Fredenburg, N T Lappas	1982
A multivariate analysis of the infrared spectra of drugs of abuse.	W W Moss, F T Posey, P C Peterson	1980
[Simultaneous determination of opioid compounds in human urine by UPLC-MS/MS].	Chang-Shu Zhan, Bao-Hua Shen, Xian-Yi Zhuo	2010
[Forensic-medical diagnostic implication of morphine levels in the blood and urine].	S V Shigeev, V V Zharov	2005
Distinction among eight opiate drugs in urine by gas chromatography-mass spectrometry.	W Nowatzke, J Zeng, A Saunders, A Bohrer, J Koenig, J Turk	1999
[Analysis of 37 drugs in whole blood by HPLC after solid phase extraction].	Chen Liang, Yu-Rong Zhang, Qi-Yun Jin, You-Mei Guo	2006
Comparison of phenyl-type columns in the development of a fast liquid chromatographic system for eighteen opiates commonly found in forensic toxicology.	R Dams, W E Lambert, K M Clauwaert, A P De Leenheer	2000

Simultaneous analysis of codeine, morphine, and heroin after B-glucuronidase hydrolysis.	M Zezulak, J J Snyder, S B Needleman	1993
Comparison of results for quantitative determination of morphine by radioimmunoassay, enzyme immunoassay, and spectrofluorometry.	V R Spiehler, D Reed, R H Cravey, W P Wilcox, R F Shaw, S Holland	1975
Considerations in the interpretation of urine analyses in suspected opiate intoxications.	B Levine, J E Smialek	1998
Solid phase extraction of morphine and its metabolites from postmortem blood.	J Gerostamoulos, O H Drummer	1998
Liquid-solid extraction of lyophilized biological material for forensic analysis. I. Application to urine samples for detection of drugs of abuse.	J R Broich, D B Hoffman, S J Goldner, S Andryauskas, C J Umberger	1971
A review of some GLD-FID derivitization techniques found useful in forensic toxicology.	G Cimbura, J Kofoed	1974
Forensic drug testing for opiates. IV. Analytical sensitivity, specificity, and accuracy of commercial urine opiate immunoassays.	E J Cone, S Dickerson, B D Paul, J M Mitchell	1992
A solid phase extraction technique for the isolation and identification of opiates in urine.	W Huang, W Andollo, W L Hearn	1992

Improved GC/MS analysis of opiates with use of oxime-TMS derivatives.	M Cremese 1, A H Wu, G Cassella, E O'Connor, K Rymut, D W Hill	1998
[Use of thin layer chromatographic analysis in forensic medical analysis of urine for opiates].	N A Gorbacheva, A M Orlova	2003
Determination of morphine and its 3- and 6-glucuronides, codeine, codeine-glucuronide and 6-monoacetylmorphine in body fluids by liquid chromatography atmospheric pressure chemical ionization mass spectrometry.	M J Bogusz, R D Maier, M Erkens, S Driessen	1997
Clinical urinalysis of drugs and alcohol in instances of suspected surreptitious administration ("spiked drinks").	Simon P Elliott, Victoria Burgess	2005
Liquid chromatography-mass spectrometry in forensic and clinical toxicology.	H H Maurer	1998
Simultaneous quantification of opiates and effect of pigmentation on its deposition in hair.	Sooyeun Lee, Eunyoung Han, Eunmi Kim, Hwakyung Choi, Heesun Chung, Seung Min Oh, Young Mi Yun, Seok Hun Jwa, Kyu Hyuck Chung	2010
The reliability of immunoassay for determining the presence of opiates in the forensic setting.	D Kimberley Molina, Vincent J Dimaio	2005
Capillary electrophoresis for the investigation of illicit drugs in hair: determination of cocaine and morphine.	F Tagliaro, C Poiesi, R Aiello, R Dorizzi, S Ghielmi, M Marigo	1993

Significance of Morphine Concentration in Bile, Liver, and Blood: Analysis of 52 Cases of Heroin Overdoses.	Isabella Mercurio, Gianluigi Ceraso, Paola Melai, Alessio Gili, Gianmarco Troiano, Fausto Agostinelli, Massimo Lancia, Mauro Bacci	2019
[Assessment of the intake of opiates (heroin, morphine, codeine and ethylmorphine) by the analysis of intermediate metabolites in the urine: which are the criteria to adopt?].	L Rivier, C Staub, C Giroud	1991
Simultaneous analysis of thebaine, 6-MAM and six abused opiates in postmortem fluids and tissues using Zymark automated solid-phase extraction and gas chromatography-mass spectrometry.	R J Lewis, R D Johnson, R A Hattrup	2005

Appendix 2 Concentrations of reconstituted solutions and calibrators

This appendix summarises the concentrations of the reconstituted lyophilised solutions and the calibrators as stated by the manufacturer.

Table 2.1: Control concentrations as stated by the manufacturer

	Level 1	Level 2
Benzoylecgonine	60 microgram/mL	300 microgram/mL
Cocaethylene	70 microgram/mL	300 microgram/mL

Table 2.2: expected concentrations for morphine and 6 monoacetyl morphine according to manufacturer's instructions

	Level 1	Level 2
Morphine	100 microgram/mL	300 microgram/mL
6 monoacetyl morphine	80 microgram/mL	300 microgram/mL

Table 2.3: control concentrations

	Level 1	Level 2
Cocaine	70 microgram/mL	300 microgram/mL

Table 2.4: calibrator concentrations

calibrator	Level 1 concentration in ng/ml	Level 2 concentration in ng/ml	Level 3 Concentration in ng/ml	Level 4 concentration in ng/ml	Level 5 concentration in ng/ml
Benzoyl-ecgonine	0	50	100	200	500
cocaethylene	0	50	100	200	500
cocaine	0	50	100	200	500
morphine	0	80	150	275	650
6-monoacetyl morphine	0	75	100	200	500

Appendix 3 Raw data for the results section

This appendix consists of the raw data obtained as GC-MS readings for each section and the calibration curves used to take the readings. The results shown are rounded to 2 decimal places, however the calculations were carried out on the true readings of the GC-MS, which are to 3 decimal places, which account for the slight discrepancies.

Results for Benzoylecgonine level 1

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	1a	70.89	79.93	75.41	9.04
	1b	77.72	75.42	76.57	2.31
	1c	73.48	74.78	74.13	1.30
Run 2	1a	84.73	85.97	85.35	1.24
	1b	80.85	70.89	75.87	9.96
	1c	91.79	84.38	88.09	7.41
Run 3	1a	88.63	105.60	97.12	16.97
	1b	85.95	75.47	80.71	10.48
	1c	96.04	81.42	88.73	14.62
Run 4	1a	71.25	76.14	73.70	4.89
	1b	85.90	85.52	85.71	0.37
	1c	68.35	65.35	66.85	2.99
Run 5	1a	75.53	120.17	97.85	44.64
	1b	75.79	114.87	95.33	39.06
	1c	74.01	76.37	75.19	2.361
total				82.44	11.18

Results for benzoylecgonine control level 2

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	2a	310.96	317.29	314.12	6.33
	2b	291.09	301.45	296.27	10.36
	2c	312.97	264.45	288.71	48.52
Run 2	2a	242.15	227.80	234.98	14.36
	2b	251.08	285.11	268.46	34.03
	2c	280.05	353.00	316.53	72.95
Run 3	2a	282.96	299.57	291.27	16.61
	2b	312.65	305.90	309.28	6.75
	2c	273.04	310.41	291.72	37.37
Run 4	2a	257.40	263.16	260.28	5.76
	2b	247.75	251.45	249.60	3.70
	2c	247.43	260.82	254.13	13.39
Run 5	2a	531.84	554.31	543.08	22.47
	2b	452.66	508.37	480.52	55.70
	2c	972.55	983.61	978.08	11.07
total				358.47	23.95

Results for cocaethylene control level 1

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	1a	77.46	73.94	75.70	3.52
	1b	69.41	64.66	67.04	4.75
	1c	70.55	74.00	72.28	3.45
Run 2	1a	76.74	80.33	78.53	3.60
	1b	69.25	68.71	68.98	0.48
	1c	75.84	79.00	77.41	3.16
Run 3	1a	83.37	80.42	81.89	2.95
	1b	87.52	83.63	85.57	3.89
	1c	82.33	77.95	80.14	4.39
Run 4	1a	71.32	74.63	72.98	3.32
	1b	73.15	70.45	71.80	2.71
	1c	69.69	67.49	68.59	2.21
Run 5	1a	83.58	89.37	86.48	5.79
	1b	77.94	83.41	80.68	5.47
	1c	71.86	69.50	70.68	2.37
total				75.98	3.47

Results for cocaethylene control level 2

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	2a	337.13	292.64	314.88	44.49
	2b	346.16	290.25	318.21	55.91
	2c	315.88	288.02	301.95	27.87
Run 2	2a	329.63	375.23	350.93	45.60
	2b	338.26	294.56	316.41	43.70
	2c	353.17	335.11	344.14	18.07
Run 3	2a	350.00	328.50	339.25	21.50
	2b	368.02	326.44	347.23	41.58
	2c	338.36	327.19	332.77	11.17
Run 4	2a	301.00	360.74	330.87	59.73
	2b	322.08	331.35	326.71	9.27
	2c	313.32	313.14	313.23	0.18
Run 5	2a	376.42	357.93	367.17	18.49
	2b	335.11	355.81	345.46	20.69
	2c	363.06	345.44	354.25	17.62
total				333.57	29.06

Results for morphine control level 1

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	1a	128.82	120.04	124.43	8.78
	1b	119.44	116.41	117.92	3.03
	1c	77.15	86.55	81.85	9.40
Run 2	1a	94.93	140.38	117.65	45.45
	1b	116.14	128.95	122.54	12.81
	1c	122.41	126.35	124.38	3.94
Run 3	1a	116.09	113.86	114.97	2.23
	1b	116.12	106.57	111.35	9.55
	1c	130.66	124.04	127.35	6.62
Run 4	1a	116.72	113.01	114.86	3.71
	1b	112.96	125.39	119.17	12.43
	1c	125.62	114.73	120.18	10.89
Run 5	1a	104.21	97.23	100.72	6.98
	1b	111.58	146.76	129.17	35.18
	1c	152.56	185.36	168.96	32.80
total				119.70	13.59

Results for Morphine level 2

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	2a	393.14	382.83	387.99	10.31
	2b	376.29	355.10	365.70	21.19
	2c	420.64	407.34	413.99	13.29
Run 2	2a	421.45	421.86	421.64	0.44
	2b	411.61	388.09	399.85	23.53
	2c	493.80	479.41	486.61	14.39
Run 3	2a	459.22	438.59	448.90	20.63
	2b	420.06	410.93	415.49	9.13
	2c	428.78	453.17	440.98	24.39
Run 4	2a	400.19	414.67	407.43	14.48
	2b	381.72	381.70	381.71	0.02
	2c	406.15	372.55	389.35	33.59
Run 5	2a	443.90	433.92	438.91	9.99
	2b	695.56	581.37	638.46	114.20
	2c	698.14	628.81	663.48	69.30
total				446.70	25.24

Results for 6 monoacetylmorphine control level 1

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	1a	88.31	71.80	80.05	16.51
	1b	72.01	75.52	73.77	3.51
	1c	25.88	20.27	23.08	5.61
Run 2	1a	24.57	64.52	44.55	39.95
	1b	47.51	93.07	70.29	45.56
	1c	52.53	57.99	55.26	5.46
Run 3	1a	73.34	62.26	67.80	11.09
	1b	54.14	53.91	54.03	0.23
	1c	64.52	48.34	56.43	16.18
Run 4	1a	48.22	59.93	54.07	11.72
	1b	55.26	71.92	63.59	16.67
	1c	56.62	56.90	56.76	0.28
Run 5	1a	71.07	79.07	75.07	8.00
	1b	46.57	63.66	55.12	17.09
	1c	90.15	88.07	89.11	2.08
total				61.26	13.33

Results obtained for 6 monoacetylmorphine control level 2

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	2a	244.15	254.00	249.07	9.85
	2b	266.10	271.75	241.93	5.65
	2c	280.37	269.91	275.14	10.47
Run 2	2a	226.60	264.37	245.48	37.77
	2b	231.67	211.26	221.46	20.40
	2c	280.60	258.26	269.43	22.33
Run 3	2a	218.86	220.79	219.84	1.91
	2b	247.19	231.79	239.49	15.40
	2c	223.22	252.23	237.73	29.01
Run 4	2a	227.99	236.09	232.03	8.11
	2b	215.03	259.86	237.44	44.83
	2c	233.56	240.88	237.22	7.32
Run 5	2a	280.81	265.83	273.32	14.99
	2b	343.53	363.16	353.35	19.63
	2c	391.68	464.46	428.07	72.78
total				264.07	21.36

Results obtained for 6 cocaine control level 1

Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	1a	76.53	74.94	75.74	1.60
	1b	81.96	258.62	170.29	176.66
	1c	70.37	75.33	72.85	4.96
Run 2	1a	86.27	87.08	86.67	0.81
	1b	79.37	98.00	88.68	18.63
	1c	81.86	80.52	81.19	1.34
Run 3	1a	74.39	70.32	72.36	4.08
	1b	69.13	65.28	67.18	3.92
	1c	74.80	68.56	71.68	6.23
Run 4	1a	270.65	256.03	263.34	14.62
	1b	69.10	65.56	67.33	3.54
	1c	69.13	62.42	65.78	6.71
Run 5	1a	Below quant.	below quant.		
	1b	834.168	872.477	853.2925	38.309
	1c	15.524	4.378	9.951	11.146
Total					

Results obtained for 6 cocaine control level 2

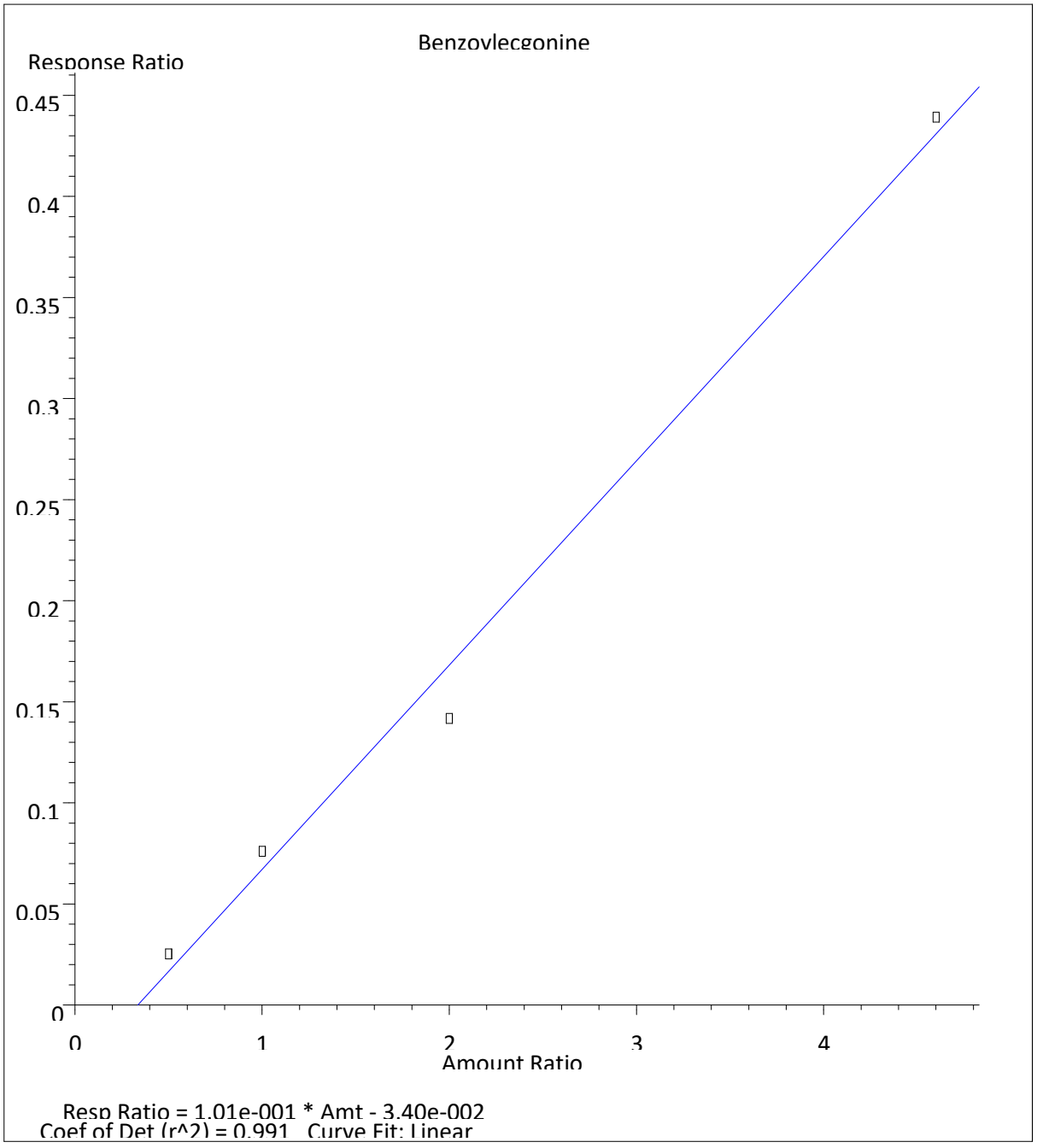
Each replicate was tested on the GC/MS two separate times, here listed as first and second reading. For subsequent tests it is the average value which was considered. The difference between first and second reading was also calculated.

Run	Replicate	First reading Concentration in ng/mL	Second reading Concentration in ng/mL	Average Concentration in ng/mL	Difference between first and second reading Concentration in ng/mL
Run 1	2a	267.00	273.41	271.70	3.41
	2b	370.49	501.99	436.24	131.51
	2c	317.14	295.63	306.38	21.51
Run 2	2a	272.31	285.80	279.05	13.49
	2b	273.40	252.49	262.94	20.91
	2c	247.39	234.48	240.93	12.91
Run 3	2a	245.80	240.59	243.20	5.12
	2b	272.70	265.56	269.13	7.16
	2c	239.69	487.32	363.50	247.63
Run 4	2a	228.26	241.13	234.69	12.87
	2b	248.18	243.34	245.76	4.84
	2c	250.34	279.77	265.05	29.44
Run 5	2a	1308.664	1256.479	1282.5725	52.185
	2b	140.922	164.134	152.528	23.212
	2c	163.954	170.005	166.9795	6.49
Total				334.71	

Appendix 4 Calibration curves used for quantification of benzoylecgonine and cocaethylene

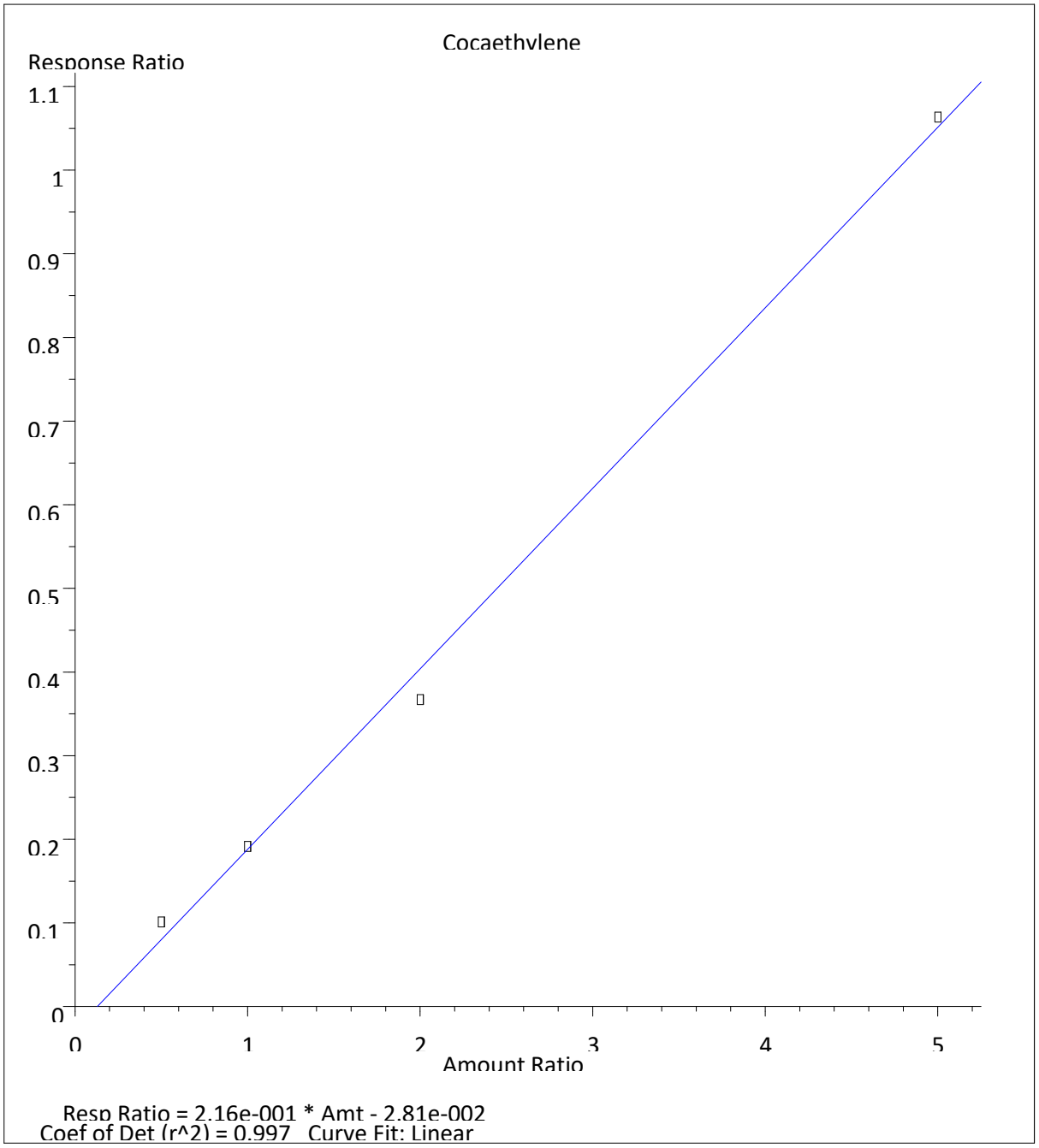
Calibration curve for benzoylecgonine

This is the calibration curve obtained from running the calibrators provided by the manufacturer for benzoylecgonine. This calibration curve was used to measure the benzoylecgonine concentration of the control runs and urine samples. The curve follows the linear equation y=mx+c, with R^2=0.991.



Calibration curve for cocaethylene

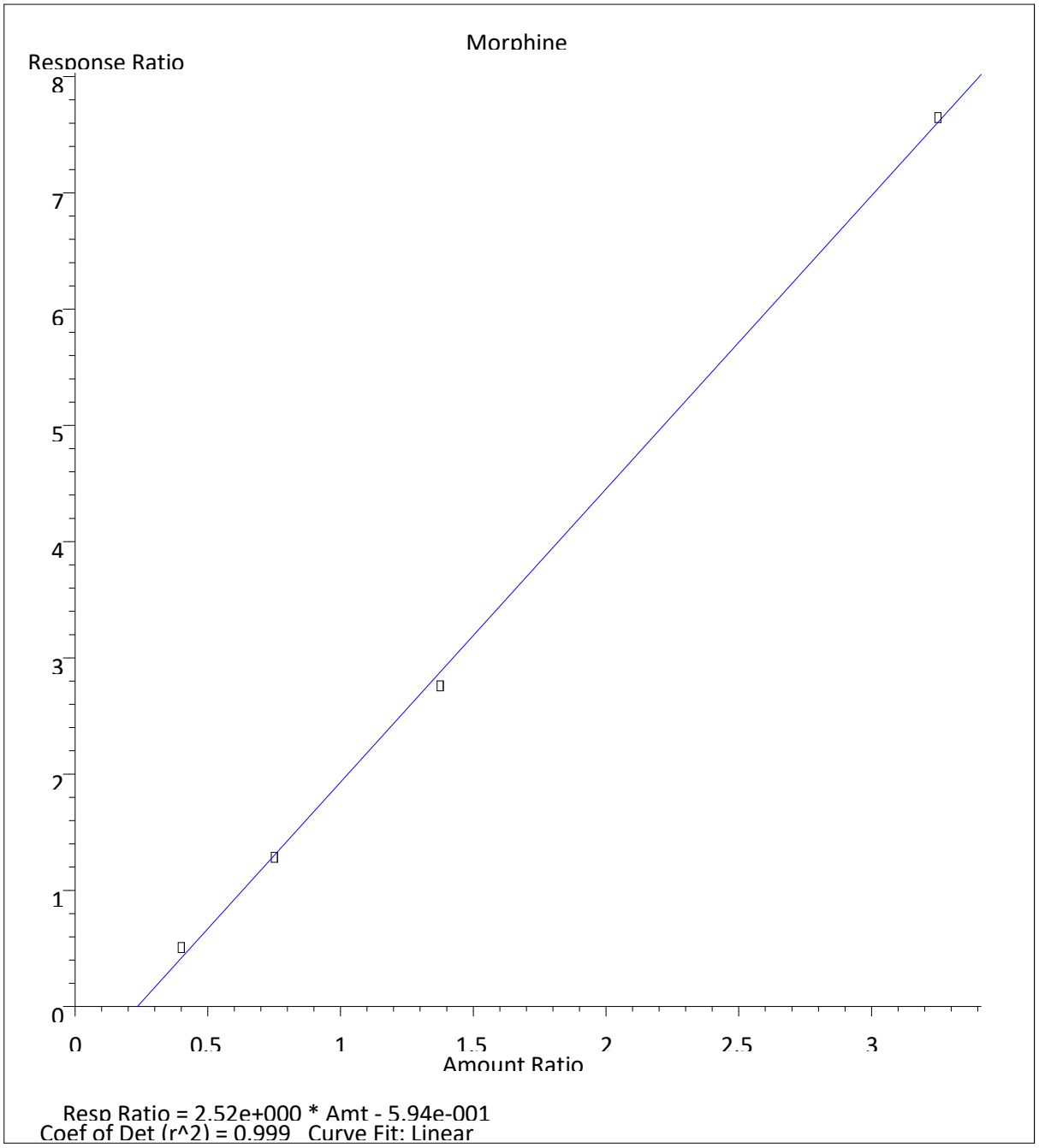
This is the calibration curve obtained from running the calibrators provided by the manufacturer for cocaethylene. This calibration curve was used to measure the cocaethylene concentration of the control runs and urine samples. The curve follows the linear equation $y=mx+c$, with $R^2=0.997$.



Calibration curves used for quantification of morphine and monoacetyl morphine

Calibration curve for morphine

This is the calibration curve obtained from running the calibrators provided by the manufacturer for morphine. This calibration curve was used to measure the morphine concentration of the control runs and urine samples. The curve follows the linear equation $y=mx+c$, with $R^2=0.999$.



Calibration curve for 6-monoacetyl morphine

This is the calibration curve obtained from running the calibrators provided by the manufacturer for 6-monoacetyl morphine. This calibration curve was used to measure the 6-monoacetyl morphine concentration of the control runs and urine samples. The curve follows the linear equation y=mx+c, with R^2=0.991.

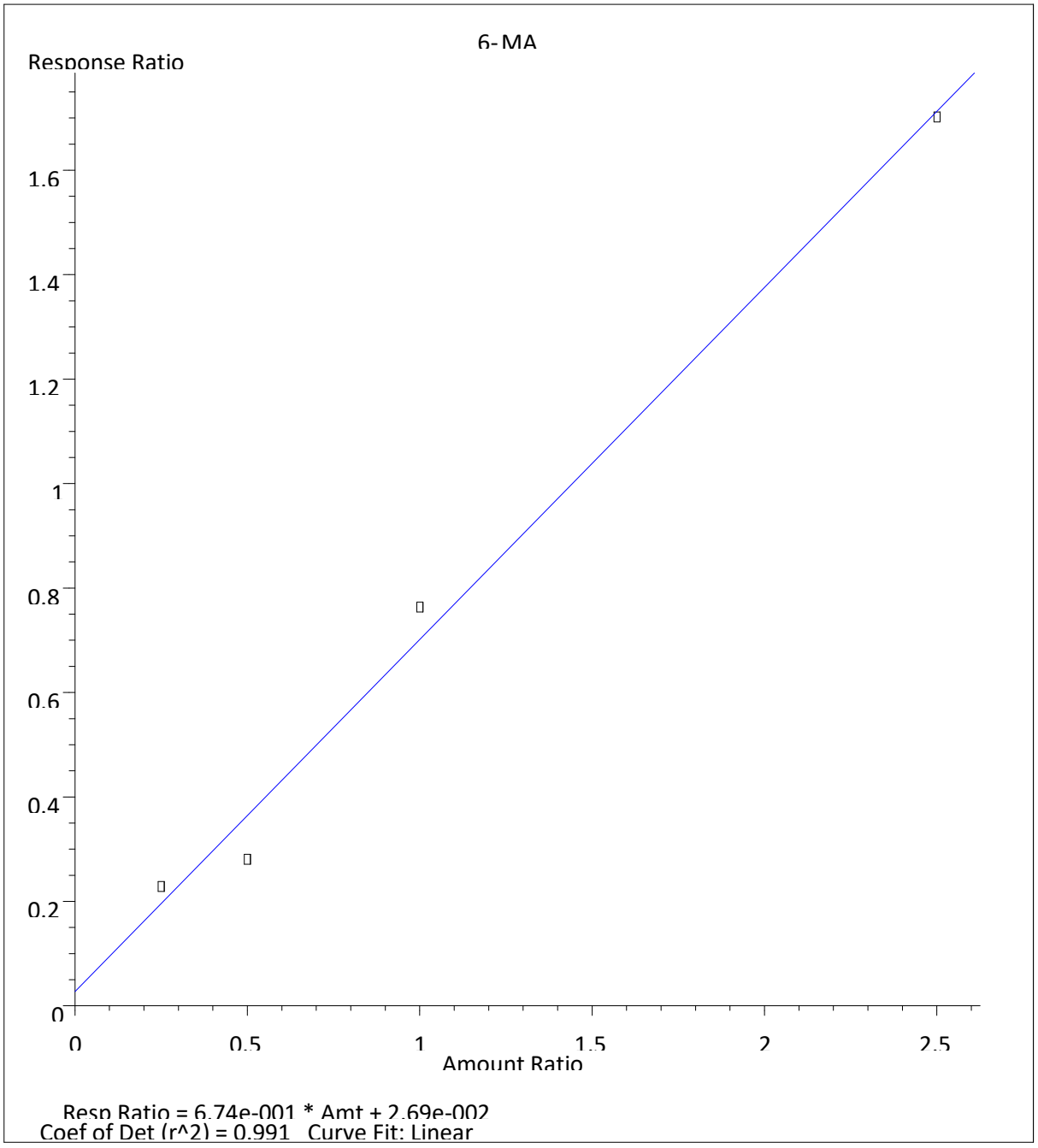
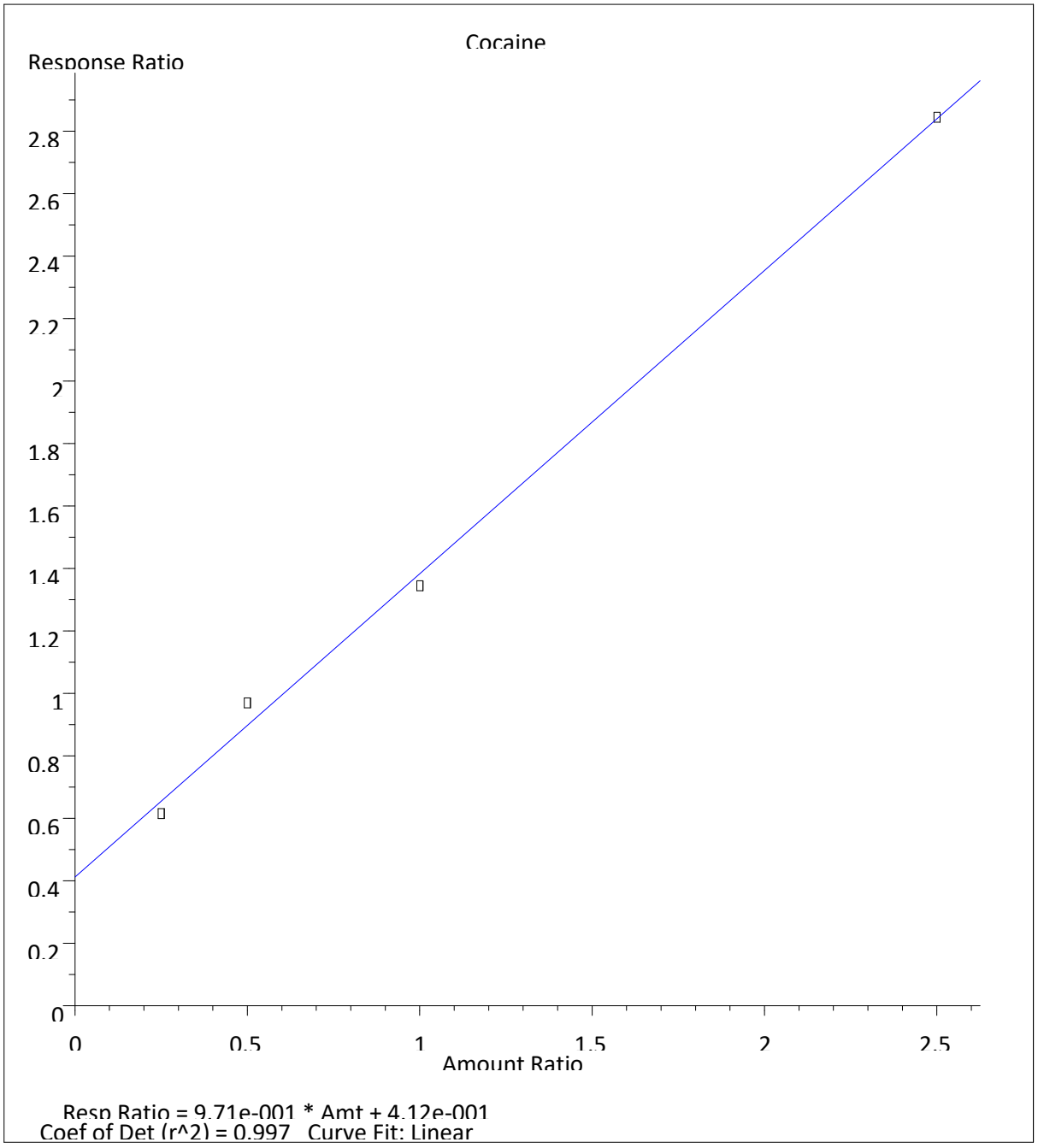


Figure 3.5: Calibration curve for cocaine

This is the calibration curve obtained from running the calibrators provided by the manufacturer for cocaine. This calibration curve was used to measure the cocaine concentration of the control runs and urine samples. The curve follows the linear equation y=mx+c, with R^2=0.997.



Annexes

L-Università ta' Malta

DEPARTMENT OF CHEMISTRY
Faculty of Science
University of Malta
Msida MSD 2080, Malta

12th January 2023

Dear Ms. Schembri:

In view of the court decree dated 9th January 2023 (see attached), whereby the Senior Magistrate Dr. G. Vella accepted the application by the undersigned for urine samples to be used for research purposes as long as points (i) to (iii) of the same application are followed, I would be grateful if you accept to be an Intermediary for this project and thus, please proceed as follows:

1. Confirm your acceptance as an Intermediary for the research project to be conducted by Ms. Fabrienne Catania.

2. With immediate effect, once FREC approval is obtained, please fully anonymise urine samples that will be used for the research by Ms. Fabrienne Catania.

3. Once anonymisation is complete, these samples are to be stored as per standard operating laboratory procedure followed by the Forensic Analysis Laboratory meaning that these same samples will be stored in a freezer in a room that is armed with an alarm, and video monitored with restricted access.

Kindly confirm this way forward in writing.

Thank you and kind regards

Dr. Godwin Sammut Ph.D. | Scientific Officer - Forensics

Associate Member American Academy of Forensic Science (AAFS); Postgraduate member Society of Toxicology (SOT)

Lecturing Department of Criminology, Department of Mental Health and Department of Clinical Pharmacology and Therapeutics

Faculty of Science, Department of Chemistry, Porta Cabin Room 25, Carpark 3 +356 2340 3157

Forensic Analysis Laboratory and Toxicology

L-Università ta' Malta

Fabrienne Catania <fabrienne.catania.17@um.edu.mt>

Request for data protection clearance

Demi Agius <dagius@advocatesprimei.com> 3 August 2023 at 17:33
To: Fabrienne Catania <fabrienne.catania.17@um.edu.mt>
Cc: Zammit Young Ingrid at MFH <ingrid.zammit-young@gov.mt>, Legal Office at MFH <legal-office.mdh@gov.mt>

Dear Ms Catania

We have no objection to approve the study titled "**Partial validation for the analysis of benzoylecgonine and opiates in urine using solid phase extraction and GC/MS** " as long as the necessary rules and terms as per the hospital's Data Protection Policy and related policies are adhered to.

Furthermore, any applicable rules, regulations, terms, and policies applicable to you or to this type of study, as may be updated from time to time by MDH, shall also apply

You are kindly also reminded that the following conditions apply

- Prior further written approval is needed before the study is presented in any local or international conference, local or abroad, or published in any way. Before publishing your study please provide us with a copy of the abstract/study as well as the name of the journal in which it will be published, for our approval.
- Further approval must be sought should you or any member of the team be requested to sign any agreement or contract in relation to the study and/or publication.
- All terms of your engagement with the hospital and/or Public Service continue to be applicable to you at all times, including in this matter.

Kindly acknowledge receipt and your agreement to the above by return email

Best regards
Demi

Dr. Demi Agius

Associate

LLB (Hons) MAdv, MA Fin Serv (Melit.)

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Ref No: MED-2023-00021

28 August 2023

Ms Fabrienne Catania
57, Sparrow,
Triq Hal Kirkop,
MQABBA, Malta

With reference to your application submitted to the Faculty Research Ethics Committee in connection with your research entitled:

Partial validation for the analysis of benzoylecgonine and opiates in urine using solid phase extraction and GC/MS

The Faculty Research Ethics Committee is granting ethical approval for the above-mentioned application.

Professor Anthony Serracino Inglott
Chair
Faculty Research Ethics Committee

il-Maġistrat,
Rat ir-Rikors;
Tilqa' t-talba b' dana peró
li t'wiehed każ jiġu segwiti
ir-rekwiżiti (i') sa' (iii') indikati
fir-Rikors.
Gabriella Vella
9.1.2023

Fil-Qorti tal-Maġistrati (Malta)

393

Dr. Gabriella Vella LL.D (Senior Magistrate)

Rikors tax-Xjenzat Forensiku Dr. Godwin Sammut Ph.D.

Re: Użu ta` kampjuni tat-tossikoloġija wara li jkunu analizzati għal-Qorti

Illum 9 ta` Jannar tal-2023

Jesponi bir-rispett,

Sabiex dejjem intejbu l-analiżi jeħtieġ li minn żmien għal-żmien isiru modifikazzjonijiet fil-metodi li jintuzaw għall-analiżi speċjalment fil-qasam tal-tossikoloġija. Bħalissa ġewwa l-*Forensic Analysis Laboratory* (L-Universita ta` Malta) għaddejja riċerka sabiex isiru modifikazzjonijiet u jitjiebu metodi u analiżi li jintużaw għall-identifikazzjoni tal-*opioids and metabolites* u *cocaine and metabolites* fid-demm u fil-*urine*. Sabiex dawn il-metodi u l-analiżi jkunu ikkonfermati li qed jaħdmu, ikun hemm bżonn li jintużaw kampjuni li diġa kienu analizzati bil-metodu li normalment jintuża.

Għaldaqstant, l-esponent jixtieq jitlob lil din Onorabbli Qorti sabiex jkun jista` juża kampjuni tat-tossikoloġija għal-dan il-għan, iżda billi jirrispetta dawn il-kriterji:

(i) L-esponent jerġa jagħmel rikors lil-Maġistrat Inkwerenti tal-każ inkwistjoni (tal-każ li se jintuża l-kampjun),

(ii) Il-każ ikun diġa sarlu l-analiżi mill-esponent għal-fini tal-inkjesta,

(iii) Illi fejn ir-rikors jiġi milquh, il-kampjuni ħa jinżammu anonimi u jintużaw biss sabiex ikun ikkonfermat il-metodu u l-analiżi l-ġdid.

Grazzi u dejjem tiegħek,

Dr. Godwin Sammut Ph.D.

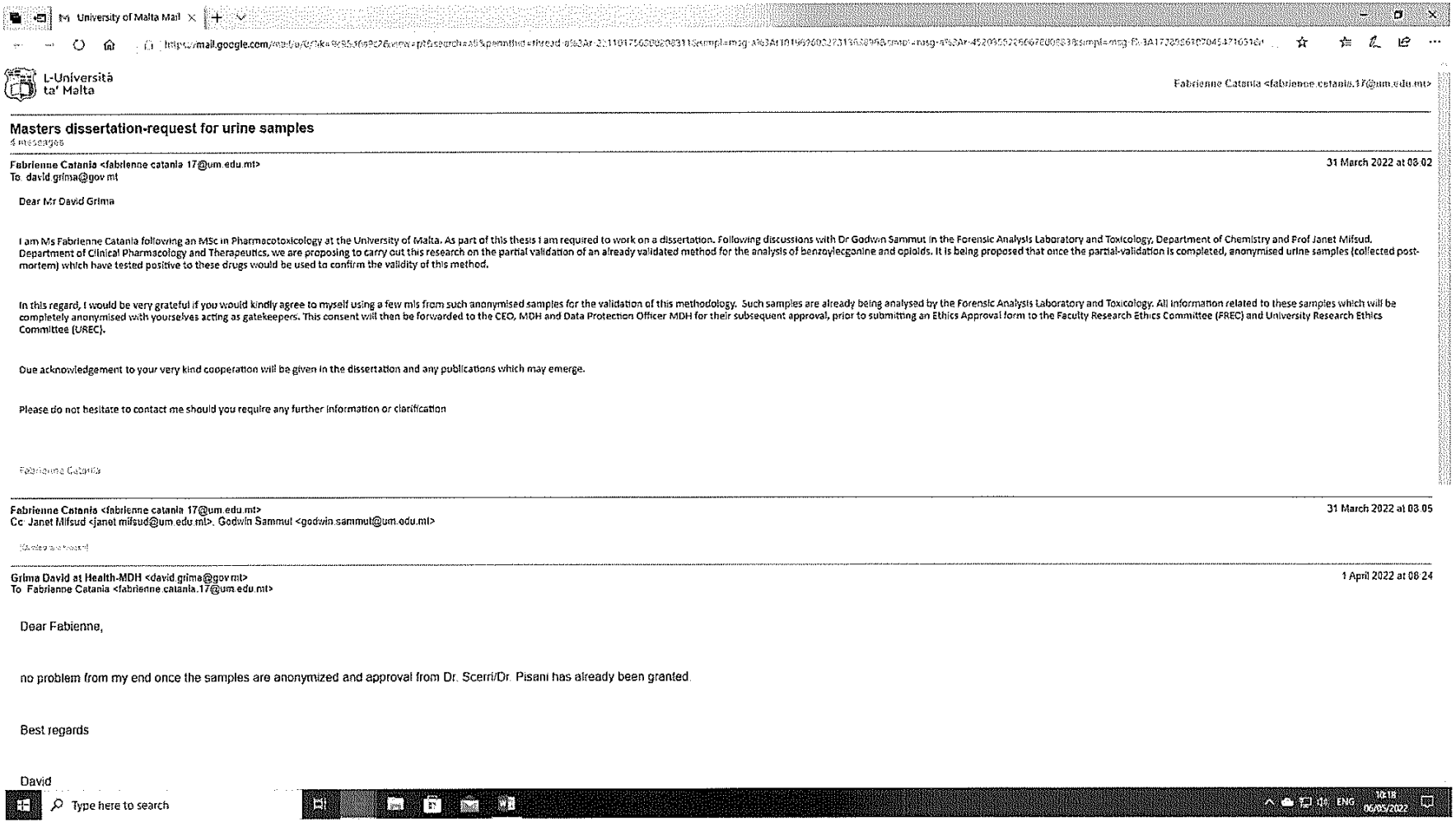
Forensic Scientist
Department of Chemistry
University of Malta

Ipprezentat minn
Dr Godwin Sammut

Illum 09 JAN 2023

Bla / b'

Audrey Bukumunti
Deputy Registrar



University of Malta Mail

L-Università ta' Malta

Fabrienne Catania <fabrienne.catania.17@um.edu.mt>

Masters dissertation-request for urine samples

4 messages

Fabrienne Catania <fabrienne.catania.17@um.edu.mt> — 31 March 2022 at 08:02
To: david.grima@gov.mt

Dear Mr David Grima

I am Ms Fabrienne Catania following an MSc in Pharmacotoxicology at the University of Malta. As part of this thesis I am required to work on a dissertation. Following discussions with Dr Godwin Sammut in the Forensic Analysis Laboratory and Toxicology, Department of Chemistry and Prof Janet Mifsud, Department of Clinical Pharmacology and Therapeutics, we are proposing to carry out this research on the partial validation of an already validated method for the analysis of benzoylecgonine and opioids. It is being proposed that once the partial-validation is completed, anonymised urine samples (collected post-mortem) which have tested positive to these drugs would be used to confirm the validity of this method.

In this regard, I would be very grateful if you would kindly agree to myself using a few mls from such anonymised samples for the validation of this methodology. Such samples are already being analysed by the Forensic Analysis Laboratory and Toxicology. All information related to these samples which will be completely anonymised with yourselves acting as gatekeepers. This consent will then be forwarded to the CEO, MDH and Data Protection Officer MDH for their subsequent approval, prior to submitting an Ethics Approval form to the Faculty Research Ethics Committee (FREC) and University Research Ethics Committee (UREC).

Due acknowledgement to your very kind cooperation will be given in the dissertation and any publications which may emerge.

Please do not hesitate to contact me should you require any further information or clarification

Fabrienne Catania

Fabrienne Catania <fabrienne.catania.17@um.edu.mt> — 31 March 2022 at 08:05
Cc: Janet Mifsud <janet.mifsud@um.edu.mt>, Godwin Sammut <godwin.sammut@um.edu.mt>

[Quoted text hidden]

Grima David at Health-MDH <david.grima@gov.mt> — 1 April 2022 at 08:24
To: Fabrienne Catania <fabrienne.catania.17@um.edu.mt>

Dear Fabienne,

no problem from my end once the samples are anonymized and approval from Dr. Scerri/Dr. Pisani has already been granted.

Best regards

David

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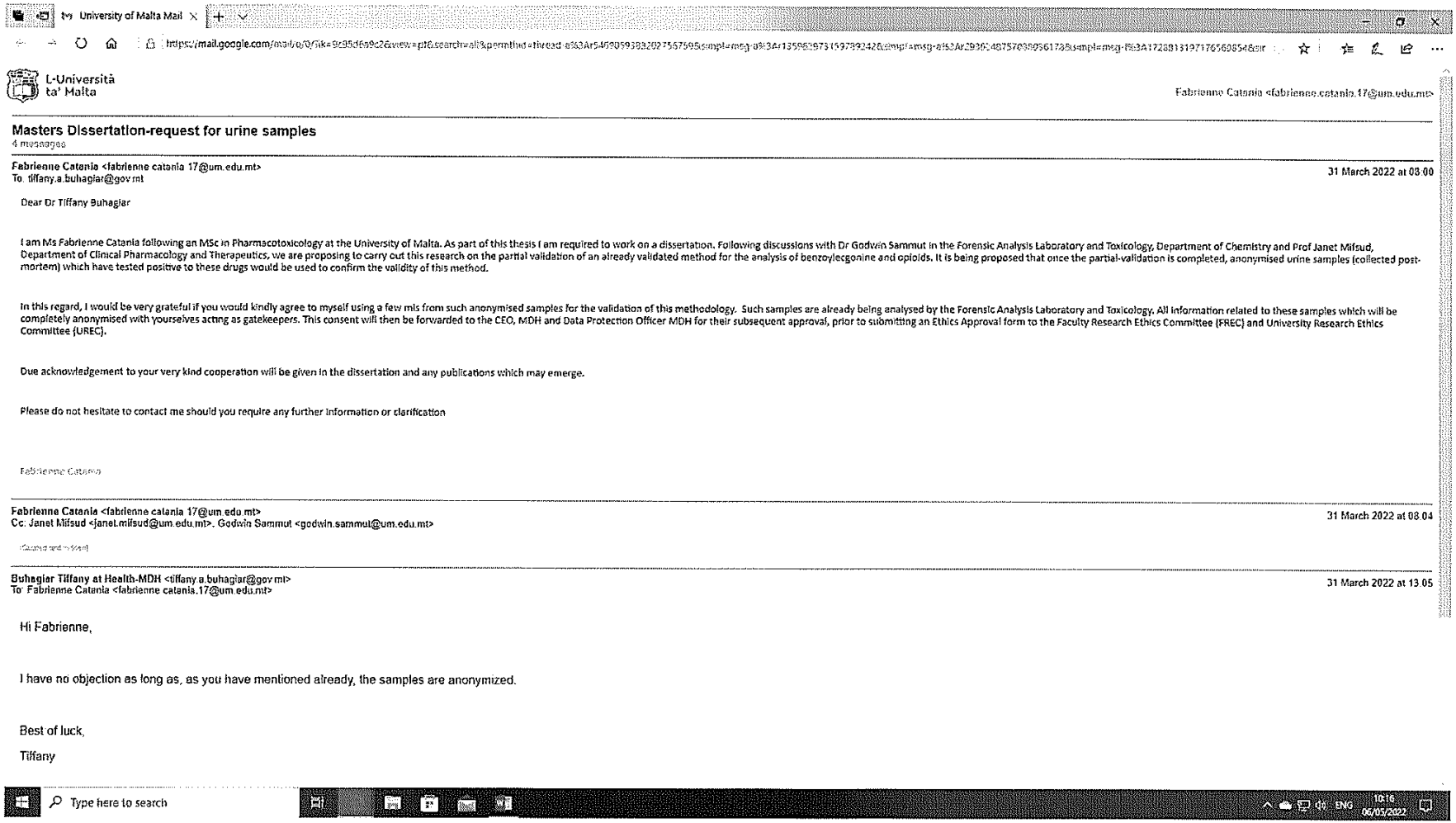

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L-Università ta' Malta

Fabrienne Catania <fabrienne.catania.17@um.edu.mt>

Masters Dissertation-request for urine samples

4 messages

Fabrienne Catania <fabrienne catania 17@um.edu.mt>
To: tiffany.a.buhagiar@gov.mt

31 March 2022 at 08:00

Dear Dr Tiffany Buhagiar

I am Ms Fabrienne Catania following an MSc in Pharmacotoxicology at the University of Malta. As part of this thesis I am required to work on a dissertation. Following discussions with Dr Godwin Sammut in the Forensic Analysis Laboratory and Toxicology, Department of Chemistry and Prof Janet Mifsud, Department of Clinical Pharmacology and Therapeutics, we are proposing to carry out this research on the partial validation of an already validated method for the analysis of benzoylecgonine and opioids. It is being proposed that once the partial-validation is completed, anonymised urine samples (collected post-mortem) which have tested positive to these drugs would be used to confirm the validity of this method.

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Please do not hesitate to contact me should you require any further information or clarification

Fabrienne Catania

Fabrienne Catania <fabrienne catania 17@um.edu.mt>
Cc: Janet Mifsud <janet.mifsud@um.edu.mt>, Godwin Sammut <godwin.sammut@um.edu.mt>

31 March 2022 at 08:04

[Quoted text hidden]

Buhagiar Tiffany at Health-MDH <tiffany.a.buhagiar@gov.mt>
To: Fabrienne Catania <fabrienne catania.17@um.edu.mt>

31 March 2022 at 13:05

Hi Fabrienne,

I have no objection as long as, as you have mentioned already, the samples are anonymized.

Best of luck,

Tiffany

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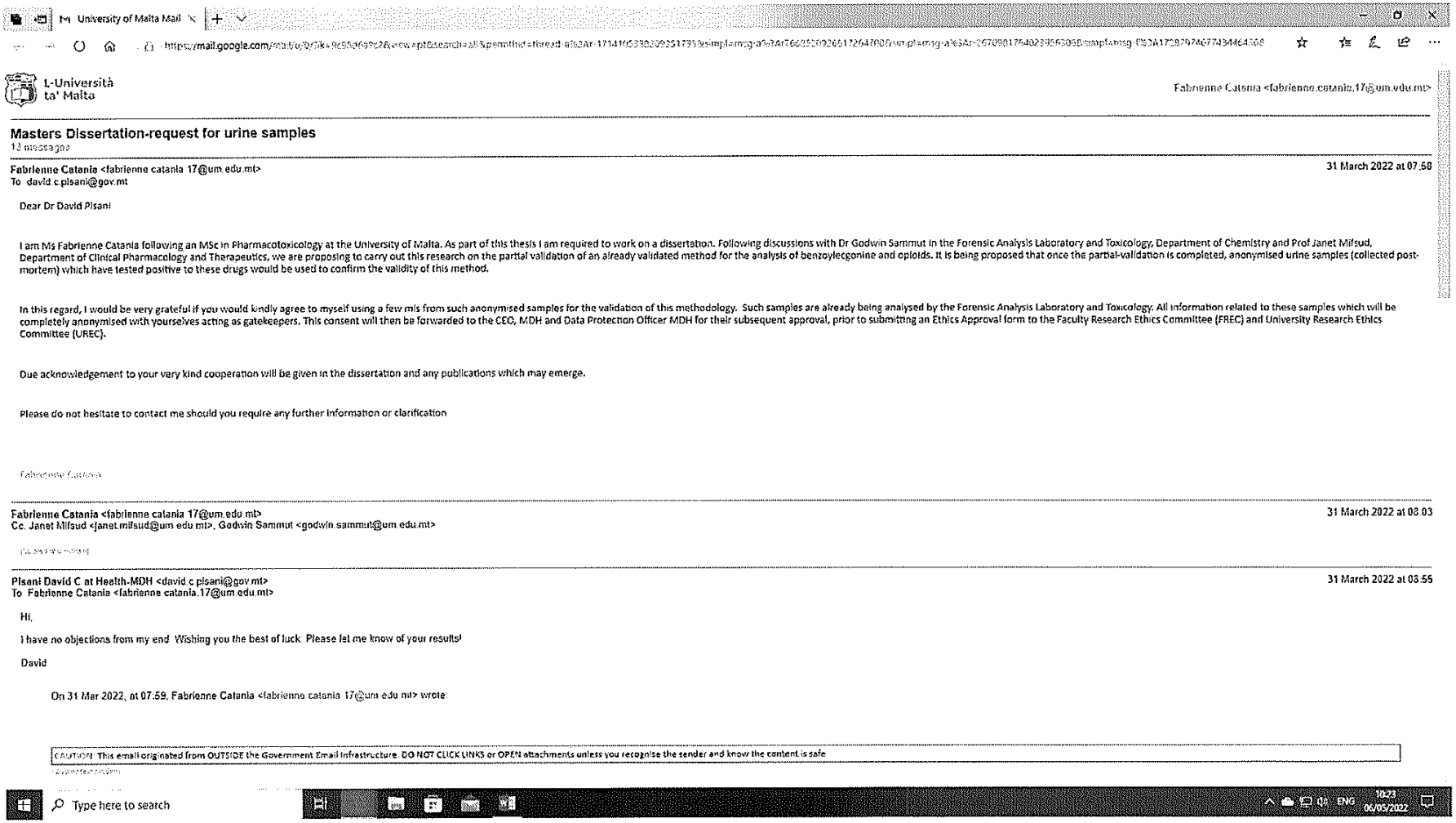

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Fabrienne Catania <fabrienne.catania.17@um.edu.mt>

Masters Dissertation-request for urine samples

13 messages

Fabrienne Catania <fabrienne.catania.17@um.edu.mt> — 31 March 2022 at 07:58
To: david.c.pisani@gov.mt

Dear Dr David Pisani

I am Ms Fabrienne Catania following an MSc in Pharmacotoxicology at the University of Malta. As part of this thesis I am required to work on a dissertation. Following discussions with Dr Godwin Sammut in the Forensic Analysis Laboratory and Toxicology, Department of Chemistry and Prof Janet Mifsud, Department of Clinical Pharmacology and Therapeutics, we are proposing to carry out this research on the partial validation of an already validated method for the analysis of benzoylecgonine and opioids. It is being proposed that once the partial-validation is completed, anonymised urine samples (collected post-mortem) which have tested positive to these drugs would be used to confirm the validity of this method.

In this regard, I would be very grateful if you would kindly agree to myself using a few mls from such anonymised samples for the validation of this methodology. Such samples are already being analysed by the Forensic Analysis Laboratory and Toxicology. All information related to these samples which will be completely anonymised with yourselves acting as gatekeepers. This consent will then be forwarded to the CEO, MDH and Data Protection Officer MDH for their subsequent approval, prior to submitting an Ethics Approval form to the Faculty Research Ethics Committee (FREC) and University Research Ethics Committee (UREC).

Due acknowledgement to your very kind cooperation will be given in the dissertation and any publications which may emerge.

Please do not hesitate to contact me should you require any further information or clarification

Fabrienne Catania

Fabrienne Catania <fabrienne.catania.17@um.edu.mt> — 31 March 2022 at 08:03
Cc: Janet Mifsud <janet.mifsud@um.edu.mt>, Godwin Sammut <godwin.sammut@um.edu.mt>

[Quoted text hidden]

Pisani David C at Health-MDH <david.c.pisani@gov.mt> — 31 March 2022 at 08:55
To: Fabrienne Catania <fabrienne.catania.17@um.edu.mt>

Hi,

I have no objections from my end. Wishing you the best of luck. Please let me know of your results!

David

On 31 Mar 2022, at 07:59, Fabrienne Catania <fabrienne.catania.17@um.edu.mt> wrote:

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