Drugs in Oral Fluid – An Evaluation of the Release of Cocaine and Cocaine Derivatives from Oral Drug Depots into Oral Fluid

Aida Ximena Merchan Otalora

A thesis submitted in partial fulfilment of the requirements of Bournemouth University for the degree of Doctor of Philosophy

May 2019

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ABSTRACT

Oral fluid (OF) drug testing has been implemented in several countries including the UK for the screening and confirmatory analysis of drugs of abuse (Wille et al. 2009, Chu et al. 2012, Vindenes et al. 2012, UK Goverment 2014). OF testing offers advantages of being non-invasive, less infectious, less likely of being adulterated and simplified collection of samples compared to other matrices such as blood. However, there are still some concerns about the interpretation of the results from OF drug testing related to variation in the concentration of drugs and/or metabolites in OF in comparison with blood. The considerably higher concentrations of some drugs in OF than their respective concentrations in blood could be explained by the release of drugs from oral drug depots into OF (Huestis and Cone 2004).

The work described in this thesis aimed at enhancing the existing knowledge on the release of cocaine and cocaine related compounds from oral drug depots into OF and evaluating alternative techniques for the detection of drugs in OF and biological tissues. To accomplish this, the kinetics of release of cocaine and cocaine derivatives were investigated using an *in-vivo* and an *in-vitro* model. The *in-vivo* study evaluated the release of cocaine and derivatives from drug depots into OF by measuring the concentration of these analytes in collected OF samples from human participants that ingested or swirled a cup of coca tea. The *in-vitro* model evaluated the release using an adapted test system for studying the transport of drugs across biological membranes, Franz diffusion cells, applied on porcine oral tissue and synthetic oral fluid. Classical and alternative techniques such as liquid chromatography and Raman spectroscopy were evaluated for the analysis of cocaine and cocaine derivatives in OF and porcine oral tissue.

The research offered new insights into the present knowledge on the release of cocaine and derivatives from drug depots into OF and presented an alternative non-invasive technique for analysing cocaine in OF and tissues. The findings of the research have also contributed to the interpretation of results from OF drug testing. The *in-vivo* release of drugs from drug depots showed differences in release profiles and windows of detection for cocaine, BZE, EME and AEME following the consumption of coca tea. Windows of detection varied between analytes and indicated that the concentration of drugs in OF was the contribution of the release of drugs from drug depots and the systemic circulation. The *in-vitro* release of drugs indicated that analytes were released into OF at different rates depending on the physicochemical characteristics of the molecules. Alternative techniques for analysing cocaine in biological matrices included the use of Raman microscopy which could detect cocaine at nanogram levels. The present research is beneficial to regulatory agencies in regard to the analysis of cocaine, the windows of detection, the false positives obtained following ingestion of coca tea and alternative techniques for on-side OF drug testing.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table 2.1 Details of gold and silver substrates. 59

Table 2.2 Substrates characterisation methods. 63

Table 2.3 Intra-day and inter-day data for analysis of cocaine in SOF using Substrate 2. Analysis conducted using a Rigaku 532 nm handheld Raman spectrometer. 84

List of Abbreviations

AEME	Anhydroecgonine methyl ester
Ag	Gold
Au	Silver
BOF	Buffered oral fluid
BZE	Benzoylecgonine
CD	Chemical reduction following by deposition
CE	Cocaethylene
COC	Cocaine
CR	Chemical reduction
DUID	Driving Under the Influence of Drugs
EME	Ecgonine methyl ester
EWDTS	European Workplace Drug Testing Society
Fe_3O_4	Iron (II,III) oxide or Magnetite
$FeCl_2$	Iron (II) chloride
$FeCl_3$	Iron (III) chloride
GC-MS	Gas Chromatography coupled to Mass Spectrometry
IP_6	Inositol hexaphosphate
LC-MS	Liquid Chromatography coupled to Mass Spectrometry
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
NaCl	Sodium chloride
NaK-T	Sodium potassium tartrate tetrahydrate
NaOH	Sodium hydroxide
NC	Norcocaine
NPs	Nanoparticles
OF	Oral fluid
PBS	Phosphate buffer saline
pK_a	Acid dissociation constant
S/P	Saliva to plasma ratio
S1-6	Substrate 1-6
SERS	Surface enhanced Raman scattering
SOF	Synthetic oral fluid
SPE	Solid phase extraction
SWGTOX	Scientific Working Group for Forensic Toxicology
UKIAFT	The UK and Ireland Association of Forensic Toxicologists

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“This thesis is dedicated to my mum for always encouraging me to follow my dreams and be happy”

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Acknowledgements

"Thank you God for giving me the strength, wisdom and ability to complete this research. I am very grateful for all the peace and joy you have giving me specially in the difficult moments"

First, I want to thank my supervisory team Professor David Osselton and Dr Sulaf Assi for the opportunity to develop this research and for the constant support and advice through the development of this PhD. I am very grateful for all I have learnt during these years. I would like to thank Alere Toxicology and Bournemouth University for funding this research project. My sincere thanks to Dr Claire George for all the help, support and insightful comments.

Special thanks to my collaborators Dr Enrique Lozano from BWTek, Gareth Barnard and David James from Thermo Scientific and Darren Machen from SciMed for providing the Raman spectrometers. I would like to thank the staff at Alere Toxicology from the Analytical services, supply chain and sales team in specific to Nicole, John, Stephen, Vaibhav, Jaspar, Selena and all the guys in the lab. I really appreciate the time you dedicated to follow up on the logistics of my study and the technical guidance you gave me on the different instruments and data processing.

This project would not have been possible without the collaboration with Dr Jorge Ariel Martinez from the Pharmacy Department at National University of Colombia. I feel more than thankful for all the time, patience, compromise and help you gave me. I am very happy to say that I not only found a great researcher but a good friend, with whom I would like to continue working in the future.

All my gratitude to my second family and large group of friends: Adri, Sonia, Mandy, Alicia, John, Duncan and my boy's group Oxala, Rashid, Alex and Roman. Thank you

for all the long talks, lunches, tears and laughs. Thank you for supporting me and balancing my life taking my mind out of the PhD.

Last but most importantly I want to thank my family. My dad Jose Publio for his immense love, trust and laughs. My aunty Luz Marina for always been there for me no matter what, couching me through life and academia with love. My partner Giulio for always making me smile and for making this PhD magical. And more specially to my mum Rosalba to whom I dedicate this thesis, for always giving me the best and encouraging me to follow my dreams and be happy. Without your support and love I would not be the person I am.

Author’s declaration

I confirm that the work presented in this thesis is my own.

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Chapter 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Oral fluid (OF) drug testing has been applied to workplace drug-testing programs, driving under the influence of drugs (DUID), drug-treatment settings and the prison service to detect drugs of abuse (Drummer 2006, Chiappin et al. 2007, Bosker and Huestis 2009, Osselton 2012, Vindenes et al. 2012). Drugs in OF have similar detection times to those found in blood, for instance, drug detection in OF could indicate recent use and the effect that the drug might have on the donor at the time the OF sample was collected.

OF offers a convenient and practical matrix for the screening and confirmatory analysis of drugs of abuse (Mali et al. 2011). The preference in use of OF over traditional matrices for drug testing (such as urine or blood) relies on its numerous advantages, which include: (1) Simplified collection of samples, as OF can be collected by non-trained personnel including the donor. (2) It is a non-invasive procedure and acquisition of samples is painless. (3) Oral secretion is considered to be less infectious than blood samples, e.g. in the case of HIV-positive samples, where manipulation of OF samples is considerably safer. (4) OF samples are less likely to be adulterated or substituted. (5) Transport and storage conditions of OF samples are less strict compared with blood samples (preservatives, temperature and time of storage). (6) There is a decrease in the cost of transport, storage and trained personnel required to supervise and collect the samples when OF is used in comparison with other matrices such as blood and urine. (Kato et al. 1993, Moore and Lewis 2003, Toennes et al. 2005, Drummer 2006, Bosker and Huestis 2009).

Although OF offers a number of advantages over other matrices, OF drug testing has its limitations with regard to the high concentration of drugs that can be found in collected

samples, which are reflected in large variations of OF to blood ratios (also described as saliva to plasma ratios S/P). This variation makes it difficult to interpret the results from OF testing and currently impossible to assess impairment from this matrix (Wolff et al. 2017). Considerably higher concentrations of drugs in OF than their respective concentrations in the circulating blood have been reported by various authors (Osselton 2002, Huestis and Cone 2004, Bosker and Huestis 2009, Gjerde et al. 2010, Vindenes et al. 2012). Originally excretion of drugs into OF was supported by the hypothesis of drugs passing from the blood to the OF based on the drug's pKa as stated by Henderson-Hasselbalch equation (Haeckel and Hänecke 1996, Kidwell et al. 1998, Spiehler 2011). However, the increased drug concentrations found in OF samples are not entirely explained by this hypothesis.

Huestis and Cone (2004) suggested that drugs can be classified into those that enter OF by passive diffusion from the systemic circulation and those that enter OF from depots formed in mouth tissues. Drugs absorbed in the oral cavity should be predominately hydrophobic, but they also need to have some hydrophilicity in order to be excreted into the systemic circulation or into the OF. If the drug is exceptionally hydrophobic, there would be a tendency for the drug to be retained in the hydrophobic components of the mucosal tissue (cell membranes) and not reach the OF or systemic circulation (Pather et al. 2008). Drugs that are found at higher concentrations than expected from their S/P ratio (theoretical value based on the Henderson-Hasselbach equation) would generally be excreted into OF from depots in the oral tissues (Spiehler and Cooper 2008). Furthermore, drugs that are orally abused through smoking (crack cocaine), sublingual absorption (fentanyl or buprenorphine), consumed as liquid preparation (methadone, morphine or coca tea) or nasal insufflation (cocaine) could create substantial oral tissue depots and

therefore have elevated S/P ratios following administration (Huestis and Cone 2004, Spiehler and Cooper 2008).

Studies on the kinetics of drugs in oral tissue and OF could contribute to the understanding of the release of drug from drug depots into OF and its potential effect on OF drug testing (Huestis and Cone 2004, Drummer 2006, Spiehler and Cooper 2008, Reichardt 2014). The experiments undertaken in this PhD thesis were conducted to increase our understanding of the release of drugs from oral drug depots into OF.

As previously mention, a drug needs to have specific physicochemical characteristics in order to diffuse and cross the buccal mucosa (Madhav et al. 2009, Steffansen et al. 2010, Bartlett and van der Voort Maarschalk 2012). Drugs that have predominant lipophilicity such as cocaine have been detected in OF at considerably higher concentrations than their respective concentrations in the circulating blood. These lipophilic drugs are more likely to form drug depots in the oral cavity (Pather et al. 2008, Reichardt 2014), which could consequently increase their concentration in OF (Spiehler and Cooper 2008). Cocaine is one of the most abused drugs worldwide (Cognard et al. 2006, UNODC 2017) and the second most widely used drug of abuse in the UK and Europe (EMCDDA 2017). Thus, cocaine was selected as the principal compound for the study of the release of drugs from drug depots into OF for this research.

1.2 COCAINE

Cocaine also named benzoyl-methyl-ecgonine and coca base are consumed as recreational drug and are natural products extracted from the coca plant (Penny et al. 2009, Biondich and Joslin 2015). The coca plant is a South American plant from the

family *Erythroxylaceae* original of the Andes area. This plant genus has approximately 260 species, from which predominant species are the *Erythroxylum coca* and *Erythroxylum novogratense* (Plowman 1979, Biondich and Joslin 2015).

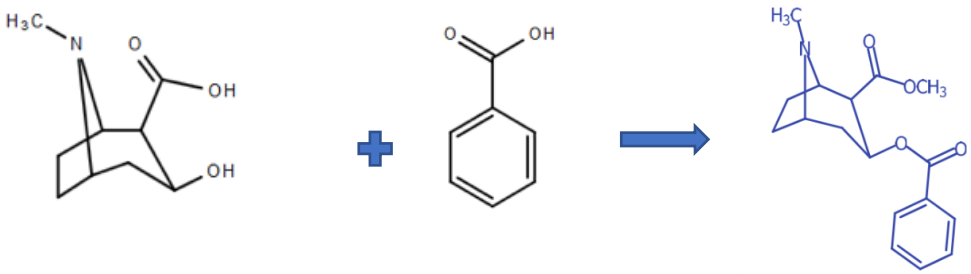
Studies on the coca plant have demonstrated that coca leaves from Bolivia, Colombia and Peru contain higher amounts of cocaine compared with other coca leaves from different countries (Moore et al. 1994, Jenkins et al. 1996, Casale et al. 2014). Cocaine concentrations by weight of coca leaf has been reported at 0.5-1.5% (Jenkins et al. 1996, Penny et al. 2009). In order to extract some of the active principles and obtain certain effects, the coca leaves are chewed, drunk as an infusion/tea or chemically extracted to obtain cocaine. Coca leaves are traditionally consumed in the Andes, to alleviate hunger, thirst, tiredness and to lessen the symptomatic relief of acute mountain sickness (AMS).

On the street, cocaine can be found as a coca paste, hydrochloride salt (cocaine hydrochloride) or as a base (cocaine base or crack-cocaine). Coca paste is the raw product resulting from the first process of extraction of the cocaine from the coca leaves. It is obtained from the maceration of coca leaves with sulfuric acid and other chemical products, e.g. alkaline organic solvents and ammonia. The result of this extraction contains approximately 40-85% of cocaine sulphate and is subsequently used in the elaboration of cocaine hydrochloride. The cocaine hydrochloride is the free base of cocaine and is commonly administered via nasal insufflation or intravenously. The cocaine base (crack-cocaine) is the product of mixing cocaine hydrochloride with a basic solution such as ammonia. When crack-cocaine is dissolved in ether, it can be volatised and subsequently inhaled by heating the solution at high temperatures (80 ºC) using propane lighters. Crack-cocaine is commonly presented as solid blocks (crystals) of 125-

300 mg with colour varying from yellow to pale rose or white and is administered via smoke (Egred and Davis 2005).

1.2.1 Cocaine Chemical Properties and Mechanism of Action

Cocaine (Figure 1.1) is a white crystalline compound with a bitter taste. It is soluble in water and reacts with acids to form salts, e.g. cocaine hydrochloride. Cocaine forms part of the tropane alkaloid group, having tropane as a fundamental core. Albert Niemann was the first person that reported the extraction of cocaine from coca leaves in 1859. Although cocaine can also be synthesised from the reaction of Ecgonine (ECG) and benzoic acid, as ECG forms esters when reacting with alcohols and acids through its OH group (Figure 1.1). The cyclic structure of ECG allows COC to generate isomers, from which L-cocaine is the most important alkaloid of coca leaf. Cocaine (molecular weight of 303.35 g/mol) is a weak base (pK_a = 8.6), highly protein bond (approximately 90%) with a melting point of 98ºC (Moffat et al. 2011).



Ecgonine Benzoic acid Cocaine

Figure 1.1 Synthesis of cocaine from ecgonine and benzoic acid.

Cocaine is a highly addictive stimulant with high toxicity (Gjerde et al. 2014). Cocaine passes through the blood-brain barrier (BBB) to reach the central nervous system (CNS), where it acts as a sympathomimetic agent, inhibiting specifically the monoamine transporters of the presynaptic membrane. In this way, the reuptake (type I) of certain

neurotransmitters such as dopamine, noradrenaline, serotonin and norepinephrine is inhibited, facilitating their accumulation in the synaptic cleft. The increased bioavailability of dopamine (the result of the inhibition of the reuptake type I) produces the feeling of euphoria and dependency when cocaine is consumed. The excess of noradrenaline generated by the consumption of cocaine is responsible for the majority of pharmacological effects and the acute complications (increased blood pressure, pupil dilatation, sudation and tremor). The inhibition of the reuptake of serotonin produces changes in its bioavailability, which is reflected in the decrease of 3-methoxy-4-hydroxyphenylglycol (MHPG) and 5-hydroxyindoleacetic acid (5-HIAA) metabolites. These processes affect the catecholaminergic and serotonergic neurotransmission, which are the basis of the mechanism of action of dependency. Norepinephrine, on the other hand, is the responsible for the changes in the vascular system: vasoconstriction and flow rate decrease. Furthermore, cocaine consumption leads to the increase in the concentration of excitatory amino acids (glutamate), which is responsible for hyperthermia and convulsions (Lizasoain et al. 2002, Ministerio de Sanidad y Consumo 2007).

1.2.2 Oral Administration of Cocaine

Cocaine from the coca leaves can be orally administered by chewing coca leaves or drinking the infusion of coca leaves (Biondich and Joslin 2015). Cocaine (cocaine hydrochloride), coca base and coca paste can be administered by different routes including drinking, snorting or smoked (Caballero and Alarcon 2000). Cone (2012) reported that the principal rout of exposure of cocaine is via smoke (63%) followed by nasal insufflation (32%) and intravenous injection (3%). Coca base (crack-cocaine) or

coca paste can also be administered by smoking or mixing the coca paste or coca base with other substances such as tobacco or cannabis.

Although the oral cavity is principally exposed to cocaine when is orally administered, nasal insufflation of cocaine hydrochloride or inhalation of the vapours produced by the burning of "crack" can also contaminate the oral cavity (Spiehler and Cooper 2008). The particles of cocaine present in the vapours or gases can pass through the nasal turbinate in the nasal cavity with help of the cilia (hair-like structures), which line the mucous membrane of the nasal cavity. The cilia move the particles trapped in the mucous and drain them into the oral cavity (Beule 2010).

1.2.3 Pharmacokinetics of Cocaine

1.2.3.1 Absorption

The absorption of cocaine following the insufflation of cocaine hydrochloride and the smoking of cocaine base was reported to be rapid, as mean plasma concentrations were obtained immediately after administration (Cone et al. 1994). Jenkins et al. (2002) confirmed the rapid absorption of cocaine by reporting mean peak plasma concentrations two minutes after smoking 40 mg of cocaine base. Zhang et al. (2012) reported that cocaine is well absorbed following nasal insufflation and that its absorption could be very rapid as psychostimulatory CNS effects are rapidly produced. Similarly, pharmacokinetic studies have demonstrated that oral cocaine is well absorbed from the gastrointestinal tract as cocaine is detected in plasma within 30 minutes of oral administration (Wilkinson et al. 1980).

Comparable results were reported by Coe et al (2018), who reported that after oral administration cocaine was rapidly absorbed and detected in plasma within 30 minutes. Coe also reported the oral bioavailability of cocaine (fraction of oral cocaine that reaches the systemic circulation) at 0.32 (100 mg oral dose) and 0.45 (200 mg oral dose) with range 0.15-0.93. These values were similar to those reported by other authors (0.2-0.6) that retrospectively calculated the bioavailability of cocaine from data across different groups of participants who received acute doses following oral or intravenous administration (Mayersohn and Perrier 1978, Wilkinson et al. 1980).

1.2.3.2 Distribution of cocaine in tissue

Several reports have demonstrated that cocaine accumulates in the body and transports across biological tissues (e.g. liver and muscle from human or pig) (Chow et al. 1985, Spiehler and Reed 1985, Poklis et al. 1987, Jeffcoat et al. 1989, Laizure et al. 2003, Othman et al. 2007, Moffat et al. 2011, Rees 2011). The volume of distribution (V_d) for cocaine were reported to range between 1 and 3 L/Kg (Moffat et al. 2011). However, little has been reported concerning the accumulation and permeability of cocaine into tissues with non-keratinised epithelia such as nasal/buccal mucosa or epithelial cell models (Bhat et al. 2001, Zhang et al. 2012, Clemons et al. 2014). Zhang et al. (2012) reported that the transport across nasal mucosa (ranged 0.2-1.0 μg/min/cm^2) was similar to the olfactory mucosa (range 0.2-0.9 μg/min/cm^2) for concentrations ranging 1-5 mM and that permeability across these tissues was dose-dependent.

Similar studies using epithelial cells (colonic T-84 monolayers) showed that cocaine transport increased linearly across these cells with the increase in cocaine concentration (100-800 ng) and that this relation did not change when the time of exposure increased from 30 to 60 minutes (Bhat et al. 2001). The rate of transport (apparent permeability

P_{app}) of cocaine across an artificial membrane of poly(vinylidene-fluoride) PVDF coated with a lipid solution (method used to mimic biological membranes such as BBB or oral mucosa) was reported at $3.66x10^{-3}$ cm/s (Clemons et al. 2014).

Different concentrations of cocaine were reported to accumulate in body tissues after administration of lethal dosages of cocaine suggesting that the transport of cocaine across tissues is more rapid in tissues with high blood flow, e.g. heart, liver and brain than in tissues with less blood supply (Poklis et al. 1987, Furnari et al. 2002, Rees 2011, Brajković et al. 2016). Poklis et al. 1987 reported high concentrations of cocaine from five related fatalities (five individuals) in kidney, brain, skeletal muscle and spleen (kidney: 39.4 ng/mL, brain: 35.3 ng/mL, skeletal muscle: 28.0 ng/mL, spleen: 26.0 ng/mL) than in adipose tissue, heart and liver (2.4 ng/mL, 5.7 ng/mL and 10.0 ng/mL respectively). Furnari et al. (2002) reported higher concentration of cocaine (based on the analysis of one individual) in bile than, brain, cardiac muscle and thigh muscle, with higher concentration of cocaine in the bile than in the cardiac muscle and thigh muscle (Furnari et al. 2002). Brajković et al. (2016) reported high concentrations of cocaine (based on one participant) in kidney and liver (21.2-24.9 µg/mL) in comparison with brain (18.9 mg/Kg), heart (9.2 µg/mL), intestine (6.1 µg/mL) and stomach (4.6 µg/mL).

1.2.3.3 Metabolism

The metabolism of cocaine is shown in Figure 1.2. Cocaine is metabolised through four pathways: (1) the enzymatic hydrolysis into ecgonine methyl ester (EME) and benzoylecgonine (BZE), which are pharmacologically inactive. BZE is formed from spontaneous hydrolysis by the hepatic carboxylesterase. The carboxylesterase hCE-1 causes the hydrolysis of cocaine to BZE by demethylation (Fleming et al. 1990, Pindel et al. 1997). (2) the hydrolysis of the benzoyl group by the action of hepatic and plasmatic

esterases (carboxylesterase hCE-2) to form EME (Pindel et al. 1997). (3) the production of EME via serum butyrylcholineterase (BchE) and (iv) the demethylation of cocaine to form norcocaine (NC) by the action of cytochrome (CYP) 450. NC is further metabolised by the CYP-450 enzyme to form N-hydroxynorcocaine. Further oxidative metabolism produce minor metabolites: *m*-hydroxycocaine, *p*-ydroxycocaine *n*-benzoylecgonine, *m*-hydroxybenzoylecgonine and *p*-hydroxybenzoylecgonine (Coe et al. 2018).

Cocaine can spontaneously hydrolyse into BZE and EME *in-vitro* and *in-vivo* at physiological temperature and pH at a rate of 4.8% (*in vitro*) of total cocaine per hour (Baselt et al. 1993, Warner and Norman 2000).

Other cocaine derivatives such as anhydroecgonine methyl ester (AEME) and cocaethylene (CE) are formed after administration of cocaine or crack cocaine. AEME is formed after crack cocaine consumption and is product of the thermal degradation of cocaine (Kintz et al. 1997). AEME is further metabolised (enzymatic hydrolysis) into anhydroecgonine. In the presence of alcohol, carboxylesterase (hCE1) reacts with cocaine to form CE via *in vivo* transesterification, where the methyl ester group is replaced with an ethyl group (Lewis et al. 2004). CE is pharmacologically active, and its activity is similar to that of cocaine (Laizure et al. 2003). It has been reported that concentrations of CE are considerably higher when alcohol has previously been consumed, thus increasing the risk of overdoses. (Laizure et al. 2003, Ministerio de Sanidad y Consumo 2007).

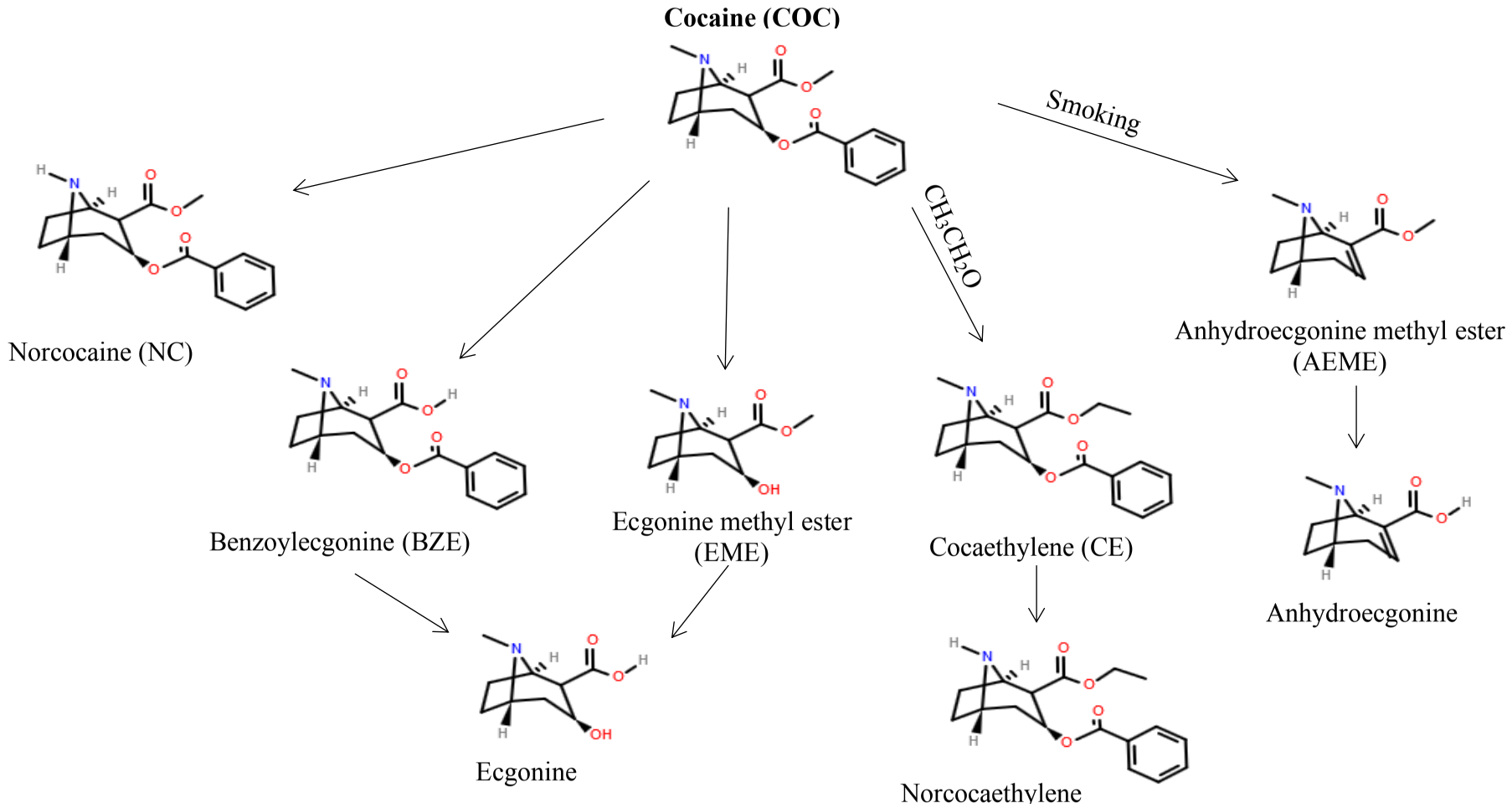


Figure 1.2 Metabolic pathway of cocaine.

1.2.3.4 Excretion

Cocaine is excreted from the body by different routes such as urine, sweat and OF (Jufer et al. 2000, 2006, Allen 2011). Cone et al. (1998) reported that 39% (intravenous), 30% (insufflation) and 16% (smoking) of a dose of cocaine was excreted in the urine within 24 hours. Cocaine and BZE were detected in sweat after two to four hours and up to 24 hours following intravenous administration of 2.1 mg/Kg cocaine hydrochloride (Kacinko et al. 2005). Cocaine and its major metabolite (BZE) were detected in OF above a concentration of 8 ng/mL (cut-off level) for up to four to eight hours after consumption of 25 mg (intravenous), 32 mg (intranasal) or 42 mg (smoked) of cocaine (Anizan and Huestis 2014). The cut-off concentration refers to the minimum concentration at which a drug (or its metabolites) must be present in a sample for the result to be considered positive (Allen 2011, EWDTS 2015, Alere Toxicology 2018). In OF the elimination half-life (t1/2) of cocaine was reported as 30 minutes following intravenous administration of 15 and 40 mg cocaine (Anizan and Huestis 2014).

A different study reported that after increasing the dose from 75 mg to 150 mg cocaine (subcutaneous administration) the time of last detection of cocaine and BZE increased from 11.5 to 32 hours for cocaine and from 17 to 47 hours for BZE respectively (cut-off levels of 2.5 ng/mL for cocaine and BZE) (Scheidweiler et al. 2010). These results indicated that the time at which a drug can be detected above its cut-off level (i.e. detection window) changes with the dosage and route of administration. The increase on detection window of cocaine in OF was also reported by different authors after giving a maximum cumulative dose of 2 g (5 doses of 25 mg per day over a period of 16 days) to human volunteers. The detection times in this study increased up to 21 and 50 hours for cocaine and BZE respectively (8 ng/mL cut-off) (Strano-Rossi et al. 2010). Jufer et al.

(2006) reported mean detection times of 85 and 93 hours for cocaine and BZE respectively, following chronic cocaine administration.

1.3 ORAL FLUID

The term OF is used to describe the fluid from the oral cavity that can be sampled, which contains a mixture of saliva and other constituents present in the mouth including (1) microbial organisms, e.g. oral bacteria, viruses, fungi; (2) cells from the oral mucosa; (3) blood and blood derivatives; (4) extrinsic substances derived from food; (5) other fluids, e.g. bronchial and nasal secretions (Table 1.1) (Kaufman and Lamster 2002, Spiehler and Cooper 2008).

Table 1.1 Components of oral fluid (Osselton 2012).

Salivary Glands	Microbes	Cells	Extrinsic substances	Blood	Other fluids
Water	Bacteria	Epithelial	Food	Micro-bleeding	Gingival fluid
Proteins	Viruses	From food	Toothpaste	Serum	Bronchial mucus
Electrolytes	Fungi		Tobacco	Cells	Nasal mucus
Other organics	-	-	-	-	-

The secretion of saliva is primarily controlled by three pairs of salivary glands that are located in mouth and throat: The parotid gland (*glandular parotis*), sublingual gland (*glandular sublingualis*) and submandibular gland (*glandular submandibularis*) (Hand and Frank 2014). An illustration of the major salivary glands is shown in Figure 1.3.

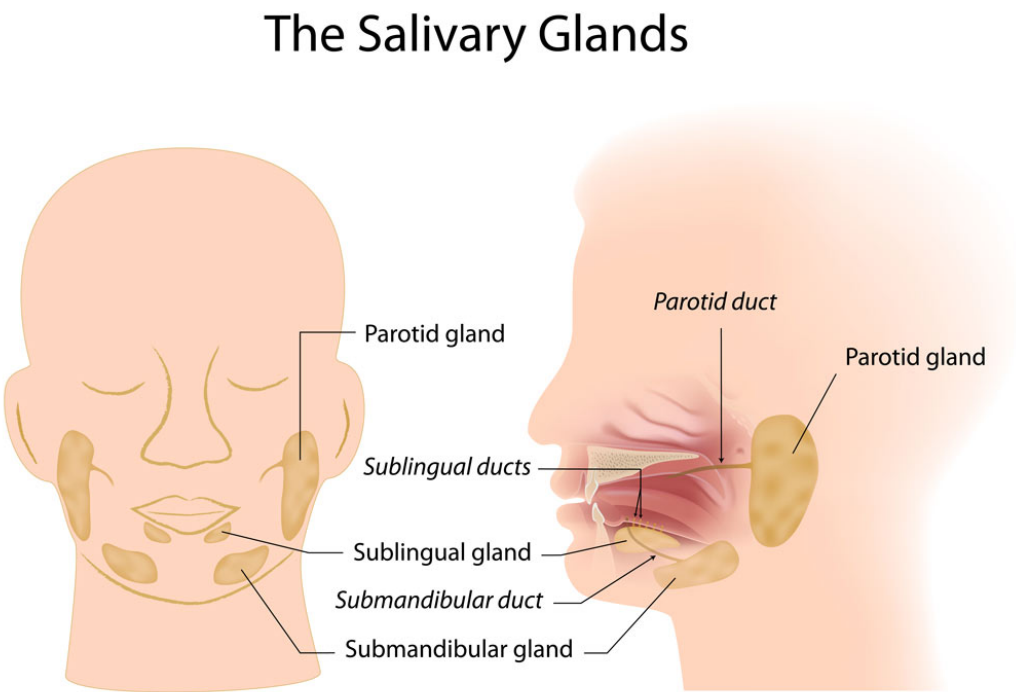


Figure 1.3 Major salivary glands with ducts that produce saliva and excrete it in the oral cavity (Biology dictionary 2019).

Saliva is also secreted by many minor salivary glands present in the oral cavity, e.g. hard and soft palates, labia and tongue. Approximately 450 – 1000 minor salivary glands are present in the oral cavity and are considered to have an exocrine function (Guzzo et al. 2010). Minor salivary glands contribute to less than 10% of total saliva, whereas major salivary glands are responsible for more than 90% of total saliva (Eliasson et al. 1996, Yoshizawa et al. 2013). The parotid gland is the largest of the major salivary glands and produces approximately 65% of the total saliva (Eliasson et al. 1996, Ferguson 1999, Hand et al. 1999, Yoshizawa et al. 2013).

Human saliva is a hypotonic biological fluid with clear, heterogeneous and slightly acidic characteristics (pH 6.0 – 7.0). It is comprised of water (99%), proteins (0.3%) and inorganic substances such as electrolytes (0.2%) (Yoshizawa et al. 2013). Electrolytes and proteins can be found at different concentrations in the saliva depending on the velocity at which the saliva passes through the ducts (Figure 1.3). Thus, higher flow rates

result in a reduced exchange of electrolytes. Changes in the content of analytes (electrolytes and proteins) and volume of saliva can be influenced by the nervous system and external factors such as (1) time of day, (2) gustatory and olfactory stimulus, (3) mechanical stimulus, (4) pain, (5) pregnancy, (6) hormone changes such as the menopause, (7) medications and (8) stress (Forde et al. 2006, Chiappin et al. 2007). Some proteins have an essential role as antibacterial and antifungal agents (e.g. lysozyme, lactoferrin, mucins), and some are important in the digestive process, e.g. α-amylase, lipase, DNase and RNase (Aps and Martens 2005).

Functions of the saliva include aiding the processes of digestion, ingestion, tasting and lubrication of oral tissues. Saliva also acts as a protective barrier against pathogenic agents. On average, individual salivation can vary from 0.3 to 0.7 mL of saliva per minute (Lenander-Lumikari et al. 1998, Ferguson 1999, Wu et al. 2008), thus producing approximately 1.0 - 1.5 L per day (Yoshizawa et al. 2013). Buffering properties of saliva rise with the high concentrations of bicarbonate in stimulated saliva. Under these conditions, the parotid gland decreases the production of saliva significantly (sublingual and submandibular glands are responsible for the production of saliva) resulting in a small volume of saliva, which is more viscous, protein-rich and can stabilise the pH of the surrounding saliva (Kaufman and Lamster 2002, Almståhl and Wikström 2003). Buffering capacity in unstimulated saliva is important for the lubrication of tissue within the oral cavity (Aps and Martens 2005, Forde et al. 2006, Chiappin et al. 2007).

Salivary glands are highly permeable and are enveloped by capillaries (Figure 1.4). These capillaries allow free transport of substances such as electrolytes and proteins into the secretory cells also called acinus cells (Guzzo et al. 2010). The primary function of the acinus cells is the excretion of fluids including the saliva into the oral cavity through the intercalated, striated and excretory ducts (Gómez de Ferraris and Campos 2002, Aps and Martens 2005).

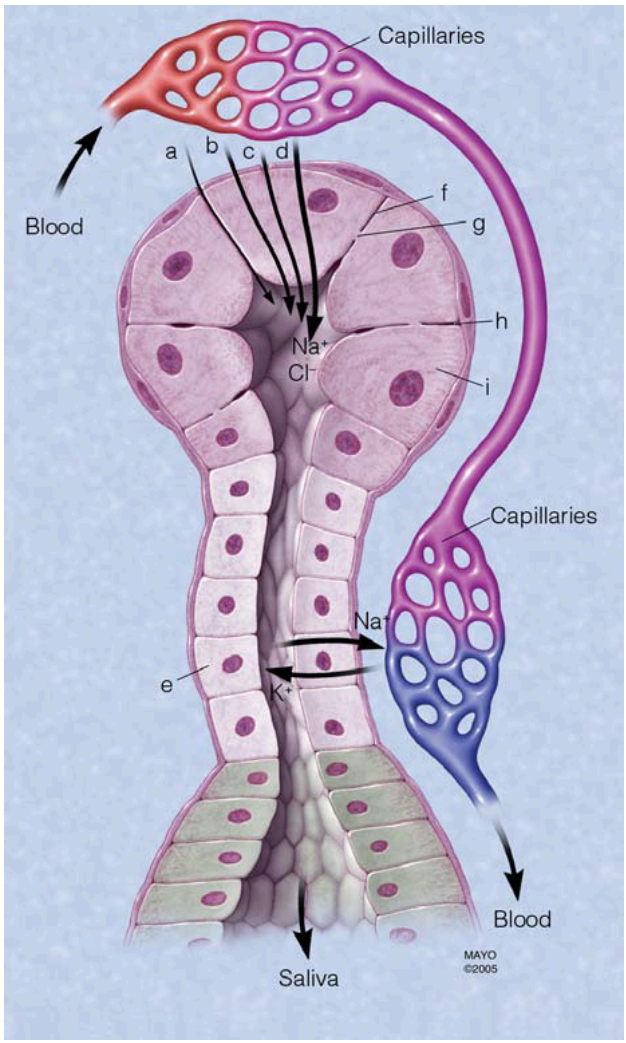


Figure 1.4 Mechanism of transport of electrolytes and proteins from the blood into salivary gland ducts. (a) Ultrafiltration, (b) active transport or passive diffusion, (c) simple filtration, (d) transepithelial movement of water along NaCl gradient via chanel proteins, (e) creation of hypotonic salivary solution via ductal Na^+ reabsorption, (f) acinal cell membrane, (g) cell membrane pore, (h) intercellular space, (i) acinar cell (Forde et al. 2006, p.45). © Quintessence Publishing Company Inc, Chicago.

1.4 HISTOLOGY OF THE ORAL MUCOSA

OF testing is conducted by the collection of OF from the oral cavity (Wolff et al. 2013). The lining of the oral cavity is the oral mucosa, the area of which (197 - 241 cm^2) accounts for approximately 80% of the total mouth cavity area, which includes the cheek and the tongue as depicted in Figure 1.5 (Naumova et al. 2013). The remaining 20% of the oral cavity correspond to the teeth. The primary role of the oral mucosa is to protect the underlying tissue from mechanical damage and to allow or prevent the absorption and excretion of exogenous substances such are drugs (Squier and Kremer 2001).

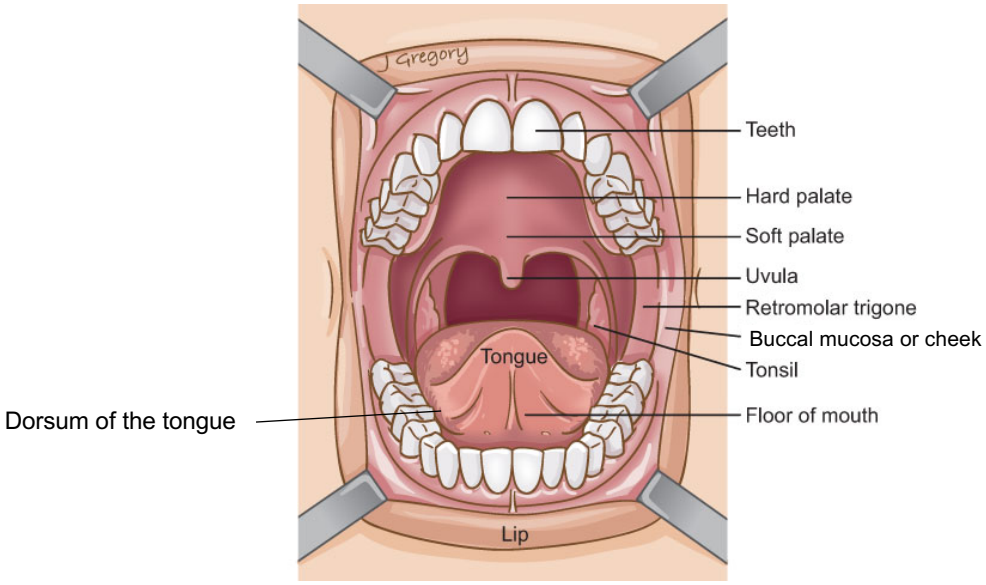


Figure 1.5 Mucosal regions in the oral cavity (Head and Neck Cancer Guide 2018). © Jill Gregory, Head & Neck Cancer Guide.

The structure of the oral mucosa, which includes the cheek and tongue is composed of a stratified epithelium which is separated from the underlying connective tissue (lamina propria) by a basement membrane (~1-2 μm thickness) (Nicolazzo and Finnin 2008). The epithelium comprises tightly packed epithelial cells whereas the lamina propria consists of fibroblasts, connective tissue, small blood vessels (capillaries), inflammatory cells (macrophages) and extracellular matrix (ECM) that aids in the fast transportation and clearance of absorbed molecules (Sonis 2004). In many regions (e.g. cheeks) a layer of

connective tissue (containing the major blood vessels and nerves) separates the oral mucosa from underlying bone or muscle as illustrated on the left side of Figure 1.6.

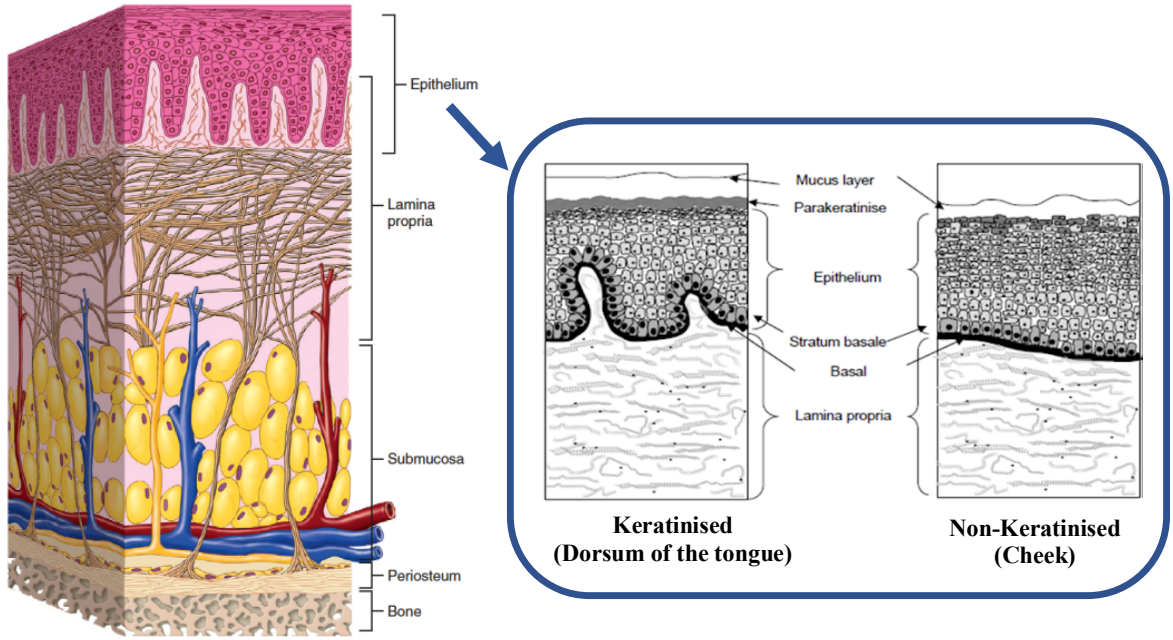


Figure 1.6 Left: Components and layers of the oral mucosa. The stratified epithelium is separated from the underlying connective tissue (lamina propria) by a basement membrane. The lamina propria and submucosa contain small blood vessels, macrophages and extracellular matrix (ECM) that aids in the transportation and clearance of absorbed molecules (Bhusnure et al. 2017, p.120). Right: The epithelium can be keratinised or non-keratinised depending on the region of the oral cavity (Vibhooti and Preeti 2018, p.2).

The epithelium of oral mucosa varies within regions in the oral cavity as shown in the right side of Figure 1.6: (1) A non-keratinised epithelium in the lining mucosa, e.g. cheek (comprising 60%). (2) A keratinised epithelium is found in the masticatory mucosa (comprising 25% of the oral mucosa). (3) Both keratinised and non-keratinised regions (specialised mucosa) are found in the dorsum of the tongue (comprising 15%) as shown in Figure 1.5 (Squier 1991, Sohi et al. 2010). Keratinized epithelium refers to an outer layer of skin which contains multiple layers of dead cells at the surface. In contrast, the non-keratinized cells are nucleated and alive.

In the cheek and under the surface of the tongue, the **non-keratinized** human buccal epithelium has 20-40 cell layers with thickness of 450-600 μm (Nielsen 2002). The

distinct layers of the non-keratinized human epithelium are (1) *Mucus layer* formed of salivary layer of approximately 70 – 100 μm. (2) *Superficial layer:* well defined flat cells that comprises 20 – 30% of the epithelium. (3) *Intermediate cell layer*: flattened cells, with thick plasma membrane and lipophilic intercellular substance excreted from the membrane coating granules (MCG). (4) *Prickle cell layer*: large cells with occurrence of MCG. (5) *Basal cell layer*: columnar cells anchored to the mechanical supportive basal lamina by hemidesmosomes. (6) *Basal lamina*: which is approximately 1 μm thick and separates the epithelium from the connective tissue (lamina propria) (Nicolazzo and Finnin 2008).

In the dorsum of the tongue the **keratinized epithelium** is composed of distinct layers: (1) *stratum corneum*: is the surface layer, formed of 10-25 rows of dead keratinocytes embedded in a lipid matrix and has a thickness of 10 – 50 μm. (2) *stratum granulosum* formed of flattened cells containing keratohyalin granules. (3) *Stratum spinosum* composed of several rows of larger spherical prickle cells. (4) The basal layer or *stratum basale* formed of a layer of cuboidal cells adjacent to the basal lamina. Both basal and stratum spinosum constitute 50 – 75 % of the thickness of the epithelium (Nicolazzo and Finnin 2008).

1.5 TRANSPORT OF DRUGS ACROSS THE ORAL MUCOSA

Oral mucosal membranes act as an efficient semi-permeable barrier system allowing diffusion of drugs, water, small molecules (electrolytes) from the systemic circulation or muscle tissue into the OF and vice-versa. Factors such as the amount of drug, degree of the drug's ionisation (pK_a), pH, size of the drug molecule, relative lipid solubility, mucosal contact time and vascularisation of the mucosal tissues controls the amount of

drug absorbed into and transported across the oral mucosa into the systemic circulation or OF (Madhav et al. 2009).

The different layers of the oral mucosa (hydrophilic mucus, keratinised layers if applicable, densely packed epithelial cell layers, basement membrane and hydrophilic connective tissue) place a barrier in the transport of drugs. It was observed that the top epithelial layer of thickness 200 μm is a major rate-limiting factor in transport kinetics of drugs (Kulkarni et al. 2010, Sohi et al. 2010). The impact of the mucus and basement layer in the transport of drugs across the oral mucosa is not well understood and may be minor compared to the inherent barrier of the epithelium (Nicolazzo and Finnin 2008). The transport of drugs within regions of the oral cavity varies significantly and is inversely proportional to keratinisation and thickness of tissue, e.g. molecules are most permeable in the buccal than the palate surfaces or the dorsum of the tongue (Figure 1.5). The higher permeability in the buccal mucosa (cheek) has been attributed to the absence of organised lipid lamellae in the intercellular spaces and the polar nature of its lipids composition (such as polar phospholipids and cholesterol esters) compared with keratinised epithelia (Nicolazzo and Finnin 2008).

The primary mechanism involved across the buccal mucosa is the paracellular route (through the spaces between the cells) by passive diffusion (Fickian diffusion) in accordance with the pH partition hypothesis (Zhang et al. 2002). Although, other transcellular mechanisms can be involved by carrier-mediated diffusion, active transport or others like endocytosis (Nicolazzo and Finnin 2008). For example, drugs such as penicillin, hormones and steroids are actively excreted through the acinus cells and ducts into the saliva (Spiehler and Cooper 2008). The passive diffusion is dependent on the physicochemical properties of the molecule (diffusion coefficient and partition coefficient). Hence, drugs require being: (1) lipophilic, (2) neutral and (3) protein free

binding in order to pass through cellular membranes. Thus, only the non-binding fraction of non-ionised drugs from the plasma is detected in OF (Sohi et al. 2010, Jones 2015).

1.6 TRANSFER OF DRUGS BETWEEN PLASMA AND ORAL FLUID

Drugs that are absorbed through the oral mucosa membranes have direct access to the systemic circulation and the OF. The relationship between the concentration of drugs in saliva and plasma was described by Rasmussen as per Equation 1. This equation was derived from the Henderson-Hasselbach equation (Equation 2) (Haeckel and Hänecke 1996). Equation 1 demonstrates that at equilibrium, the saliva to plasma ratio (*S/P*) is dependent on: (1) The concentration of drug in saliva (*S*). (2) The concentration of drug in plasma (*P*). (3) The dissociation constant for basic drugs (pK_b) or acidic drugs (pK_a). (4) The pH of the saliva (pH_s). (5) The pH of the plasma (pH_p) and (6) the fraction of drug bounded to saliva (f_s) and plasma proteins (f_p).

Equation 1. Rasmussen Equation for the saliva to plasma ratio.

Acidic drugs: $\frac{S}{P} = \frac{[1+10^{(pH_s - pK_a)}]}{[1+10^{(pH_p - pK_s)}]} \frac{f_p}{f_s}$ Basic drugs: $\frac{S}{P} = \frac{[1+10^{(pK_d - pH_s)}]}{[1+10^{(pK_d - pH_p)}]} \frac{f_p}{f_s}$

S/P: Saliva to plasma ratio; S: Concentration of drug in saliva; P: Concentration of drug in plasma; pK_b: basic drugs; pK_a: Dissociation constant for acidic drugs; pH_s: The pH of the saliva. pH_p: pH of the plasma; f_s: Fraction of bounded drug to saliva; f_p: Fraction of bounded drug to plasma proteins.

The Henderson-Hasselbach equation (Equation 2 - left) describes the pH as a measure of acidity in a chemical system using pK_a, where the pH is dependent on the pK_a and their concentration of acid [HA] and conjugate base [A^-]. An alternative form of this equation using the pK_a is given by the Heylman-Lardinois equation (Equation 2 - right), where [B] and [BH^+] are the concentration of the base and its conjugate acid respectively.

Equation 2. Henderson-Hasselbach and Heylman-Lardinois equations.

Acid: $pH = pKa + log\frac{[A^-]}{[HA]}$ Basic: $pOH = pKb + log\frac{[BH^+]}{[B]}$

pK_a: Dissociation constant for acidic drugs; [HA] Concentration of acid [A^-]: Concentration of conjugate base; [B]: Concentration of base; [BH^+]: Concentration of conjugate acid.

1.7 ELEVATED ORAL FLUID TO BLOOD DRUG RATIOS

In general, the concentration of drugs in OF are higher than corresponding concentrations in plasma (Forde et al. 2006). Elevated S/P ratios (>100 fold) have been reported for cocaine after smoking 40 mg cocaine base, with concentrations of cocaine ranging 15,852-50,480 ng/mL in saliva and 46-291 ng/mL in plasma after smoking (Jenkins et al. 1995). Fiorentin et al. (2017) reported mean cocaine concentration in OF (39 ± 70 ng/mL) five times higher than in plasma (8.2 ± 18 ng/mL) from 124 cocaine/crack users. Similarly, Scheidweiler et al. (2010) reported median values of cocaine maximum concentration in OF (1092; 406-3006 ng/mL) four times higher than in plasma (305; 109-434 ng/mL).

Furthermore, cocaine has been reported at high concentrations (ranging 1.3-3.1 μg/mL) in OF following oral administration after doses of 25-150 mg (Kato et al. 1993, Kidwell et al. 1998, Bosker and Huestis 2009). Concentrations in the range of 0.4-2490 ng/mL cocaine and 0.4-12100 ng/mL BZE in OF were reported after chronic administration of cocaine (Cone 2012). Data from the Forensic Science Service showed mean cocaine concentrations of 1191 ng/mL (33-3537 ng/mL) in OF (Osselton et al. 2001). These concentrations were significantly higher than the concentration that can be found in blood following cocaine drug overdose (1.1-98.1 μg/mL) (Karch et al. 1998, Fineschi et al. 2002).

At the lower pH of normal saliva (pH = 6.7), which is slightly acidic, many of the illicit drugs including cocaine will be ionised and leading to ion trapping in the OF (Allen 2011). This ion trapping occurs because of the decreased diffusion into blood resulting from ionisation of the molecule (DePriest et al. 2015). Since saliva presents a pH lower than the plasma, the S/P ratio for acidic drugs (pK_a < 5.5) or highly bound molecules is generally inferior to one. Neutral molecules (pK_a 5.5-8.5) have a S/P ratio near to 1.0 that does not vary with the salivary flow rate. Basic drugs and drugs that do not bind highly to proteins are ionised at saliva pH and are ion trapped in the OF producing S/P ratios greater than 1.0 (Spiehler and Cooper 2008).

For drugs with pK_a between 5.5 and 8, the S/P ratio may vary depending on the flow rate of saliva and therefore its pH (Schramm et al. 1992). The pH of saliva has been proved to be inversely proportional to the saliva flow (Dawes and Jenkins 1964). When the flow is low, sodium is less absorbed by the salivary ducts and so an accumulation of sodium produces an increase in the salivary pH. Hence, unstimulated saliva has higher pH, reaching values of up to pH 8. An example of the variation of S/P ratio with the salivary flow was reported for cocaine, with S/P ratios ranging between 3.0-9.0 when the salivary pH varied between 5.0-7.8 following intravenous (IV) dosages of 25 mg (Kato et al. 1993). The S/P ratio might also be influenced by the route of consumption, for example, snorting or smoking of drugs such as cocaine would lead to buccal contamination and therefore much higher S/P ratios (Allen 2011).

1.8 DRUG DEPOTS

Huestis and Cone (2004) initially suggested that drugs that are found at higher concentration in OF are excreted into the OF from drug depots in the oral tissues. This hypotesis was then supported by other authors (Drummer 2006, Spiehler and Cooper

2008). In 2014, Reichardt proved this hypothesis by showing (immunohistochemical staining) that drugs such as cocaine and heroin could accumulate into porcine tongue tissue following exposure to the drug in solution or simulated smoking, thus, confirming the formation of drug depots in oral tissues.

Reichardt's study showed that the staining in the porcine tongue tissue increased with the increase in drug exposure (Figure 1.7). The amount of cocaine/heroine deposited in the tissue that is proportional to the strength of the staining was subsequently confirmed by quantitative analysis of the tongue tissue using liquid chromatography coupled to mass spectrometry (LC-MS). The results of the quantitative analysis showed that cocaine concentration increased from 6-25 ng/mL (100 ng/mL dose) to 55-274 ng/mL (10,000 ng/mL dose), confirming the relation between the dose exposed and the amount of drug depot formed in the porcine tongue tissue. Comparison between the staining obtained following cocaine and heroin, showed that staining following heroin exposure was more intense than cocaine exposure. Therefore, indicating that there was a higher amount of heroin deposited into the tongue tissue than cocaine, which could be explained by the higher lipophilicity of heroin in relation to cocaine.

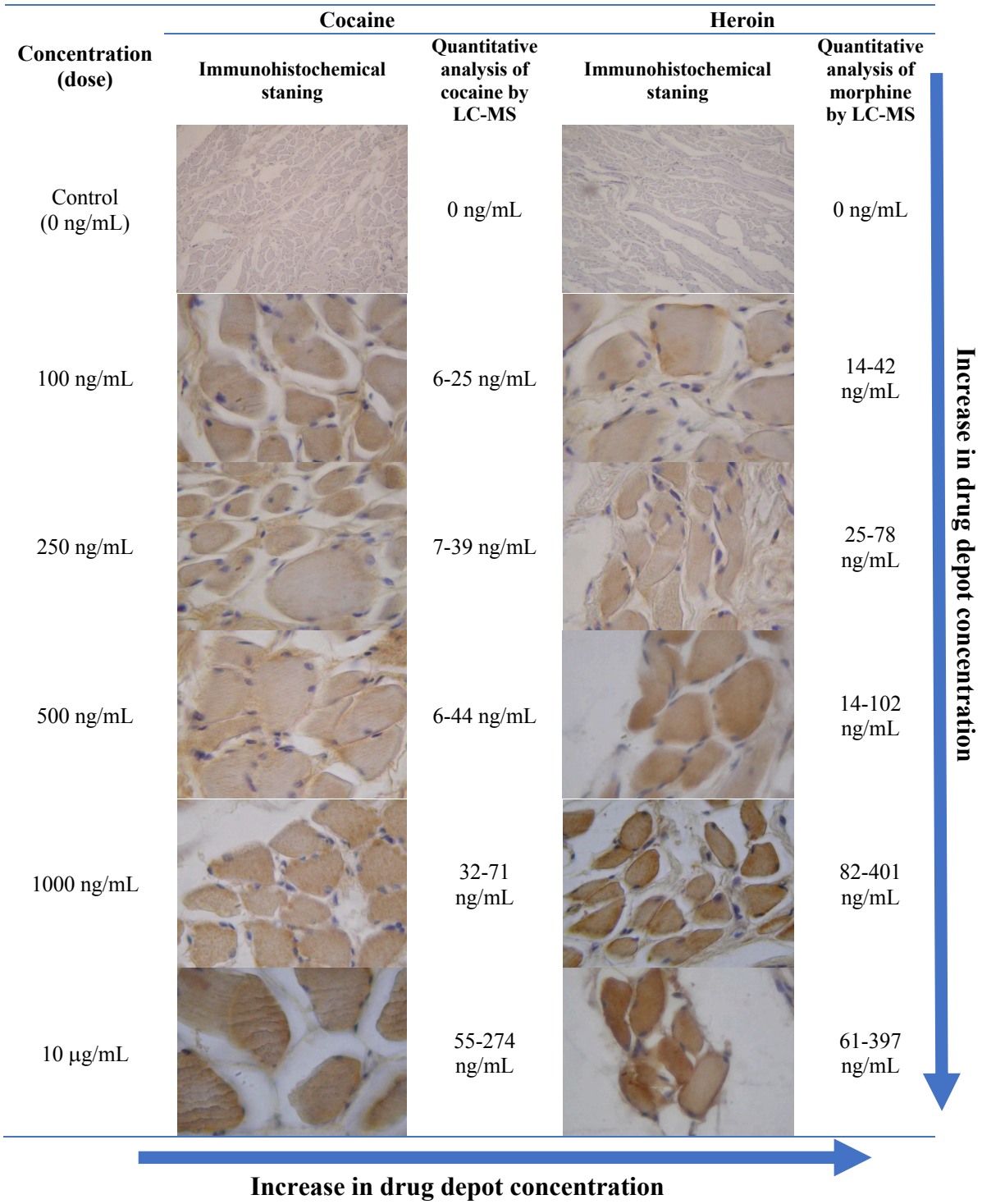


Figure 1.7 Immunohistochemical staining for cocaine and heroin in porcine tongue tissue following exposure of 100-10000 ng/mL of the drug. Images at magnification x65 (Reichardt 2014, p 183-188).

The results reported by Reichardt also included a study on the release of drugs from tongue tissue, where tongues were exposed to either 100 ng/mL or 1000 ng/mL of cocaine or heroin and then washed for 1 hour, 6 hours, 24 hours or 48 hours in artificial saliva with continuous mechanical stirring. The outcome of this study showed that cocaine was

detected for up to 24 hours post-exposure whereas morphine was detected for up to 48 hours post exposure. These results indicated that cocaine and heroin were being released from drug depots into OF over time (Figure 1.8) and could interfere with the interpretation of drug concentrations in OF when investigating S/P ratios (Reichardt 2014).

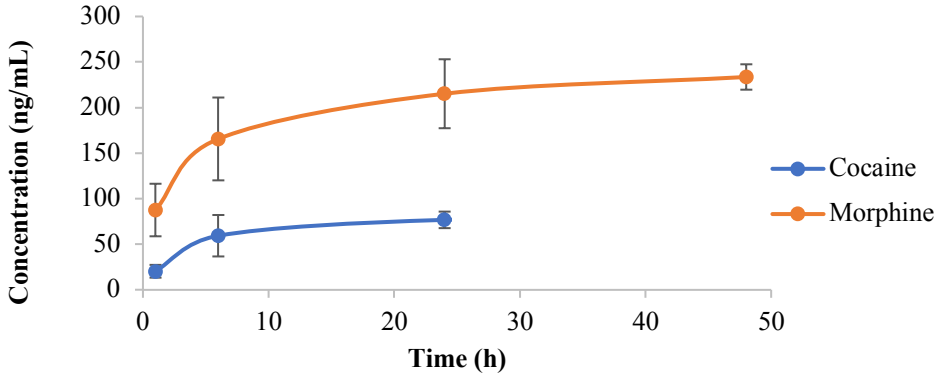


Figure 1.8 Cumulative amount of cocaine and morphine in OF following the interaction to porcine tongue tissue exposed to 1000 ng cocaine or heroin. The graph was plotted based on data reported by Reichardt (2014).

1.9 MODELS EMPLOYED TO ASSESS DRUG DIFFUSION

Study on the diffusion of drugs through biological membranes is commonly conducted by the use of *in vivo* and/or *in vitro* models, although mathematical models are also used (Bolger et al. 2002). While *in vivo* models are more appropriate for assessing the bioavailability of a drug, *in vitro* models are more commonly used for preclinical compound screening, elucidation of the mechanism of transport across the oral mucosa and assessment of compound permeability (Nicolazzo and Finnin 2008).

1.9.1 *In vivo* models to assess diffusion of drugs

One of the *in vivo* methods used to evaluate the absorption of drugs in the buccal mucosa is the test of Beckett and Triggs (Beckett and Triggs 1967). In this test, a known volume of a drug solution is introduced into the oral cavity, swirled around for a specific

period of time and then expel it. The expelled solution is finally analysed to determine how much drug was absorbed in the mucosa. The advantages of this test are the time of the study and the use of single participants, although kinetic profiles cannot be obtained.

Beckett and Triggs test was then modified by various authors. Dearden and Tomlinson (1971) introduced a factor that corrects for the production of OF (Dearden and Tomlinson 1971). Tucker (1988) modified the test by collecting samples of the swirled solution from the oral cavity every few minutes without removing the whole solution. Thus, allowing the study of the absorption kinetics on a single participant (Tucker 1988). Additionally, other authors have reported the addition of marker compounds to the swirling solution, such as phenol red, to account for salivary dilution and accidental swallowing of the solution (Nicolazzo and Finnin 2008).

Other *in vivo* methods used to determine the absorption and diffusion of drugs into the oral cavity include the use of perfusion systems, which can be attached to the oral tissues of both animals and humans (Tsai 2003, Tunblad et al. 2004, Bansal and Ajay 2012). This method consists of perfusing a specific amount of drug through the cell. The amount of drug disappearing from the perfusate accounts for the amount of drug absorbed by the tissue. Quantification of the drug in either OF or plasma has also been included in this method to evaluate the diffusion of drugs such as nicotine through the oral mucosa (Adrian et al. 2006).

1.9.2 *In vitro* models to assess diffusion of drugs

In vitro methods commonly involve the use of diffusion cells fitted with suitable membranes, e.g. porcine skin, that operate under atmospheric conditions. These models are often used to determine the kinetics of a drug across a specific tissue and to evaluate

the barrier nature of a particular biological tissue (Nicolazzo and Finnin 2008). *In vitro* models are commonly used instead of *in vivo* models because (1) they can offer comparative diffusion results (Nicolazzo and Finnin 2008, Castro et al. 2016). (2) Variables such as temperature, pH and drug concentration can be easily controlled. (3) In cases where human tissue is not available, animal models can be used instead, which reduces cost and ethical considerations (Patel et al. 2012).

The most common diffusion cells are the Franz-type diffusion cells (Figure 1.9) (Nicolazzo and Finnin 2008, Bartosova and Bajgar 2012, Castro et al. 2016). These cells are normally used to assess *in vitro* transport of compounds across the buccal mucosa (Ceschel et al. 2002). In this system, the buccal mucosa is placed in between two chambers, and buffer solutions with and without the addition of a test compound are added to the chambers. The amount of compound that has permeated the tissue over time is measured by analysing samples periodically collected from the chambers (Bartosova and Bajgar 2012).

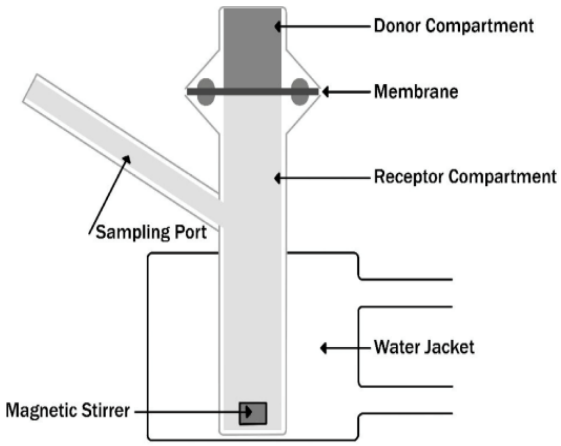


Figure 1.9 Franz type diffusion cell. (Bartosova and Bajgar 2012, p.4673)

In the Franz cell system, a donor solution containing the drug is applied to the apical surface of the membrane to initiate the absorption of the drug. The receptor compartment is filled with a receiver solution suitable for the study (such as PBS) that does not induce

any alteration to the barrier properties of the membrane (Bartosova and Bajgar 2012, Castro et al. 2016). The receiver solution should be maintain at 37 °C in order to mimic *in vivo* skin surface temperature (Nielsen 2002). Additionally, it should not exceed 10% of the donor concentration in the receiver solution (saturation should be avoided) as this maintains a concentration gradient between the donor and receiver solution (Bartosova and Bajgar 2012). To aid the maintenance of sink conditions throughout the experiment, stirring of the receiver solution is employed (Brodin et al. 2010).

Ideally, human buccal (cheek) mucosa should be used to study buccal diffusion *in vitro*, however, lack of availability of human tissue, as well as national and international ethical considerations make the use of human tissue difficult. Instead, cell culture models and animals have been employed to mimic the skin barrier properties (Shrestha et al. 2015). Porcine buccal tissue is often preferred as an alternative to the human buccal mucosa for *in v*itro studies (Nielsen 2002) (apart of primates) because of its similarities in non-keratinised buccal epithelium and biochemical properties (Gray and Yardley, 1975; Jacobi et al., 2007). Prove of this is the similarities between the diameter of the external epithelial layer and values of permeability for water between pig buccal mucosa (thickness: 772 ± 150 μm; permeability: 634 ± 60 cm/min) and human buccal mucosa (580 ± 00 μm; permeability: 579 ± 22 cm/min), which were reported to be very similar (Squier et al. 1996). Based on these results and many others, it has been recommended that permeability across buccal mucosa be conducted using porcine buccal mucosa (Nicolazzo and Finnin 2008).

Checks of the integrity of the buccal mucosa are commonly conducted during diffusion or permeability studies to guarantee that no damage to the membrane took place during the transport, preservation or after permeability studies (Pather et al. 2008). Several studies have been reported on the use of biological media and/or temperature conditions

for the preservation of tissue and its biological bioavailability. It has been reported that porcine buccal mucosa retained its integrity when stored with a preservative (Phosphate buffer saline PBS pH7, Kreb's bicarbonate ringer solution, HEPES buffer or HBSS – Hank's balanced salt solution) at 4 °C for 24 hours (Kulkarni et al. 2010). Furthermore, the buccal mucosa can also be stored for a more extended time at -20 °C following sectioning of the tissue without losing its integrity (Michaud and Foran 2011).

Regulatory authorities such as The Food and Drug Administration (FDA) Guidance 2000 have proposed guidelines for the determination of *in vivo* and *in vitro* permeability of drugs (specifically for the gastrointestinal tract). These guidelines suggest that excised human or animal tissue be used and that for the purpose of permeability evaluation viable or non-viable tissues would be suitable (van der Bijl and van Eyk 2003).

When drugs are being evaluated, the amount of drug applied in the donor compartment should be (1) an infinite dose (i.e. > 10 $mg.cm^2$), typically used when analysing the fundamental permeation behaviour of a test molecule or (2) a finite dose (i.e. 2 - 10 $\mu g.cm^2$) typically used to mimic the application of a topical dose (Howes et al. 1996). The kinetic permeation or diffusion profile is obtained by sampling the receiver fluid at defined time points and quantifying the amount of drug in the receiver fluid using accurate and sensitive methods, such as high performance liquid chromatography (HPLC) (Castro et al. 2016).

1.9.3 Mathematical models

The passive transport of molecules across a biological barrier can be described by mathematical models, e.g. zero and first order models. These mathematical modelling can be fitted on experimental data to determine physical parameters, such as the drug

diffusion coefficient (Dash et al. 2010). The release of molecules in solutions and molecular transport across biological barriers can follow different release patterns (Brodin et al. 2010). These patterns can be described by the release of drugs at a slow zero or first order rate or the release of an initial high amount of drug followed by a slow release of zero or first order.

Generally, the transport of molecules across a biological barrier is a multifactorial process, however, a simplified model has been proposed to help understand the transport process (Barry, 1983). The main mechanism involved in the transfer of molecules across the oral mucosa was described by Fick's first law in 1855 (Fick 1855, 1995). Fick's law proposes the concept that a solute will move from a region of high concentration to a region of low concentration across a concentration gradient and can be described by the Equation 1.3.

$$J = \frac{Q}{At} = D\frac{\Delta C}{h} \qquad \textbf{(Equation 1.3)}$$

Fick's equation relates the flux (J) in stationary state to an amount of compound (Q) that is transported across a barrier with area (A), over a period of time (t) with a constant concentration gradient (ΔC), a diffusion coefficient in the barrier (D) and a path length (h). This model assumes that the barrier is a pseudo-homogeneous membrane, which characteristics do not change during the compound transfer process. The slow transport across the oral mucosa or release of drug (from the mucosa) can be represented by different equations:

1.9.3.1 Zero-order model

This model describes the slow transport/release of drugs independent of the initial drug concentration. In Equation 1.4, Q is the amount of drug transported/released in time (t),

Q_0 is the initial amount of drug being transported/released and K_0 is the zero-order transport/release constant expressed in units of concentration time (μg/cm^2/h). The constant K can be obtained from experimental data by plotting the cumulative amount of drug transported/released versus time (cm/h).

$$Q_t = K_t t + Q_0 \qquad \textbf{(Equation 1.4)}$$

1.9.3.2 First order model

This model describes the slow transport/release of drugs and the absorption and/or elimination of some drugs (Dash et al. 2010) that is directly proportional to the drug concentration embedded in the matrix. In Equation 1.5 and 1.6, C is the amount of drug transported/released in time t, C_0 is the initial concentration of drug and K is the first-order transport/release constant expressed in units time (h^{-1}). The constant K can be obtained from experimental data by plotting the log cumulative amount of drug transported/released vs. time.

$$\frac{dC}{dt} = -K_c \quad \text{or} \quad logC = logC_0 - \frac{K_t}{2.303} \qquad \textbf{(Equation 1.5 and 1.6)}$$

1.9.3.3 Higuchi model

This model describes drug transport/release as a diffusion process based on the Fick's law and describes the diffusion in terms of the thermodynamic activity of the permeant compound (Higuchi 1961). When it is assumed that the concentration of a compound on the basolateral side is insignificant compared to the concentration in the matrix or apical side (sink conditions), the maximum rate of diffusion per unit time (J_{max}) is proportional to the thermodynamic activity of the compound and not its concentration (Equation 1.7).

$$J_{max} = D\frac{S_S}{h} \qquad \textbf{(Equation 1.7)}$$

In Equation 1.7, *D* is the diffusion coefficient of the compound, S_s is the maximum solubility of the compound and *h* is the barrier thickness. According to this equation, the flux of a compound from saturated conditions is constant, regardless of the saturated concentration in a given vehicle because all saturated solutions have a thermodynamic activity of one (Bronaugh and Maibach, 1989). Higuchi's model assumes that mass transfer occurs under sink conditions, the drug diffusivity is constant, drug diffusion takes place only in one direction and the application vehicle does not affect the barrier (Higuchi 1961). The Higuchi model equation is given by:

$$f_t = Q = A - K_c\sqrt{\frac{D\delta}{\tau}(2C - \delta C_s)C_s t} \qquad \textbf{(Equation 1.8)}$$

Q is the amount of drug released in time *t* per unit area *A*, *C* is the drug initial concentration, *Cs* is the drug solubility in the matrix media and *D* is the diffusivity of the drug molecules (diffusion coefficient) in the matrix substance, δ is the porosity of the matrix and τ the tortuosity (Tortuosity is defined as the dimensions of radius and branching of the pores and canals in the matrix) (Costa and Sousa Lobo 2001). A simplification of Higuchi's model is given by Equation 1.9. In this equation, K_H is the Higuchi dissolution constant (Higuchi, 1961). The constant K can be obtained from experimental data by plotting the cumulative amount of drug released vs. square root of time.

$$f_t = Q = K_H\sqrt{t} \qquad \textbf{(Equation 1.9)}$$

1.9.3.4 Korsmeyer-Peppas model

This model can be used as a decision parameter between the Higuchi and zero order models (Ciurba et al. 2014). Although this model is generally used to analyse the release of dosage forms, when the release mechanism is not well known or when more than one

type of release take place (Steffansen et al. 2010). This model assumes that the transport/release occurs in a one-dimensional way, the calculation of *n* is based on the portion of the release curve where $M_t/M_\infty<0.6$ and the system width–thickness or length–thickness relation be at least 10. The release mechanism is a function of the diffusion exponent *n,* where values of $n = 0.5$ suggests a Fickian diffusion (elimination rate as a function of time $t^{-0.5}$). Values between $0.5 < n < 1.0$ supports an anomalous non-Fickian transport (elimination rate as a function of time t^{n-1}). For n = 1.0, the release mechanism is represented by a case-II, zero order model (elimination rate is independent of time)(Costa and Sousa Lobo 2001).

$$\frac{M_t}{M_\infty} = at^n \qquad \textbf{(Equation 1.10)}$$

1.9.3.5 Quantitative Structure Property Relationship (QSPR) models

The Quantitative Structure Property Relationship (QSPR) models are used to determine the release coefficient (k_p) using algorithms derived from the molecule's octanol-water partition coefficients and molecular weight (Flynn 1990). The most commonly used QSPR model is the one developed by Potts and Guy (1992). This model correlates the log permeability coefficient ($\log k_p$) with the log partition coefficient of the drug in octanol water ($\log P_{oct/w}$) and its molecular weight (MW).

$$\log k_p = -6.3 + 0.71 \log P_{oct/w} - 0.0061MW; \text{ with } R^2 = 0.67 \text{ and } n = 93 \qquad \textbf{(Equation 1.11)}$$

1.10 MECHANISM OF RELEASE OF DRUGS FROM ORAL TISSUE INTO ORAL FLUID

The mechanism of the release of cocaine or other drugs from oral tissues into OF still unknown but it could be similar to the mechanism of action of drugs crossing the blood-

brain barrier (BBB). The similarities are based on the fact that drug molecules need to successfully pass across the hydrophobic BBB into the hydrophilic central nervous system to exert a pharmacological function (Johnston et al. 2016). In order to detect cocaine in OF the molecules need to cross barriers such as the oral mucosa. However, the high hydrophobicity of molecules such as cocaine could make the drug be sequestered by the lipophilic bed in the oral membranes and not reach the hydrophilic OF. As a result, cocaine molecules must somehow partition between the lipophilic (e.g. mucosa) and hydrophilic (aqueous OF) medium in order to be released into the OF.

Studies developed on the transport of molecules such as cocaine across the BBB have been developed to understand how small lipophilic molecules cross the BBB (Geldenhuys et al. 2012, Johnston et al. 2016). These studies suggested that the transport of molecules across biological membranes depend on the structure and the hydration of the molecule. The results reported by Johnson et al. (2016) suggested that (1) cocaine can adopt a complex ring structure, where the rings would include water molecules, which help confer lipophilicity to the cocaine molecule. (2) Cocaine is highly lipophilic even when it is protonated. (3) Cocaine is still readily water-soluble in a more closed conformation and (4) Cocaine can easily achieve both lipophilicity and hydrophilicity simultaneously without having to adopt a different conformation in either physical regime or having to go through a protonation-deprotonation reaction to effectively permeate the BBB. Based on Johnson's study, the association of water with the hydrogen bonds in cocaine could add diffusion across the BBB and therefore the oral mucosa.

1.11 RAMAN SPECTROSCOPY

Raman spectroscopy is a vibrational spectroscopic (inelastic scattering) technique being used as novel approach for screening of drugs of abuse in biological matrices such

as OF and tissue (Dronova et al. 2016, Wolff et al. 2017). In Raman scattering, the molecule retains some of the energy of the scattered photon, which results in nuclear movement in the intramolecular atoms. The scattered photon is then released with a slightly changed energy, and therefore wavelength. Each chemical bond requires a unique energy to vibrate, thus a fingerprint can be built up of the chemicals present in the sample by collecting the scattered photons with varying wavelengths. Raman spectroscopy provides detailed information on specific constituents (such as drugs) within a complex biological matrix, e.g. OF and tissues (Williams and Sebastine 2005, Farquharson et al. 2011, Dana et al. 2015).

Raman analysis offers advantages over other analytical techniques such as LC-MS or immunohistochemistry: (1) it is a non-invasive, non-destructive technique where (2) minimal sample preparation is required. (3) There is no need for toxic chemicals or direct exposure to the sample. (4) It has high chemical specificity; therefore, molecular information can be obtained without the need of chemical staining or labelling. (6) Raman spectra can be collected in few minutes. (7) Raman spectroscopy in combination with microscopy is very powerful for imaging biological samples, e.g. individual cells. (8) In combination with visible or near-infrared light it reduces the absorption of water, therefore analysis of biofluids and tissue can be obtained at near-physiological conditions. (9) Raman spectroscopy can take advantage of the advanced optical fibres, miniaturised lasers and other photonic devices. For this reason, there are hand-held and portable instruments in the market which allow measurements to be taken in real time (Movasaghi et al. 2007, Fernandes de Oliveira et al. 2012, Radzol et al. 2012, Sharma et al. 2012, Kong et al. 2015).

1.11.1 SERS - Surface Enhanced Raman Spectroscopy

Surface Enhanced Raman Spectroscopy (SERS) has been developed to enhance the Raman signal for up to 10^{13} – 10^{15} times and overcome the majority of drawbacks of conventional Raman spectroscopy such as low sensitivity (Feng 2015). SERS enhancement occurs when a molecule is in close proximity to a metal nanoparticle (NP) or metallic surface with plasmonic properties (able to form a dipole that produces oscillation of free electrons). The enhancement of the Raman signal occurs when two theoretical processes take place: (1) An electromagnetic enhancement where an interaction between the incoming radiation and the plasmon resonance produce an enhancement in the local electric field. (2) A chemical enhancement where a bond is formed between the metallic surface and the molecule under examination that increases the molecular polarizability, giving an enhancement in the signal (Sharma et al. 2012).

In SERS analysis, the detection process is identical to normal Raman analysis following excitation of the plasmon resonance and generation of the SERS signal. A long-pass filter is used to absorb or reflect any Rayleigh scattering while allowing for transmission of the Raman signal, and a spectrograph and detector are used to image Raman spectra across a wide spectral region.

In the last decade, the development of new substrates (NPs with plasmonic properties) has increased. The most common substrates used in SERS analysis are gold (Au) and silver (Ag) NPs, which can vary in structure (plasmon resonances and a range of average enhancement factors) and SERS activity (Sharma et al. 2012, He et al. 2017). The development of NPs with plasmonic properties is very important to obtain reproducible and robust results, and a whole area of research (nanotechnology) focuses on the synthesis and development of these NPs. Synthesis of NPs can be either chemical or physical:

Chemical synthesis takes place by the reduction of metal salts, micro-emulsions, thermal decomposition and electrochemical synthesis; whereas physical methods include pulsed laser ablation, chemical vapour deposition, microwave irradiation, supercritical fluids and gamma radiation amongst others (Herrera et al. 2013).

Identification of analytes in liquid or solid matrices, including drugs in biological matrices have been reported using SERS substrates (Farquharson et al. 2011, Salehi et al. 2013, Barnett and Rathmell 2015, Dana et al. 2015, Wrona et al. 2015, Yang et al. 2015). After complexation with SERS substrate samples containing cocaine were detected in the range of 2 ng/mL to 10 μg/mL (Farquharson et al. 2011; Dana et al. 2015; Feng 2015). In this case, the substrates used were silver, gold or a mixture of silver and gold NPs obtained by precipitation of silver (I) oxide, reduction of silver nitrate to silver ions or seed growth of silver and/or gold (Table 1.2).

Table 1.2 Summary of methods reported for the analysis of cocaine in OF by SERS.

Drug	Method	LLD (ng/mL)	Volume of specimen (uL)	Excitation wavelength (nm)	Reference
Cocaine/BZE	SERS[a]	50	-[c]	785	Farquharson et al. 2011
	SERS[a]	125	1000	785	Dana et al. 2015
	SERS	29	50	-	Yang et al. 2015
	SERS[a]	39,000	-	-	(Barnett and Rathmell 2015)

COC: Cocaine; BZE: Benzoylecgonine; SERS: Surface Enhanced Raman Spectroscopy. a: Handheld Raman spectroscopy; c: Unavailable information; LLD: Low limit of detection.

1.11.1.1 Silver substrates synthesised via chemical deposition

This substrate has been used for the analysis of liquid and solid state samples and therefore can be used for the analysis of cocaine in OF and tissue samples (Wrona et al. 2015, 2017). Examples of this are the analysis of butylated hydroxyl-anisole (BHA) in edible and essential oils (LOD 5% BHA in oil) (Wrona et al. 2015) and assessment of the

oxidation degree of oxo-biodegradable plastics (Wrona et al. 2017). SERS analysis was conducted by applying test solution on the surface of the NPs or depositing the NPs directly on the surface of the solid sample.

Silver substrates can be obtained using the Tollens reagent, this reagent uses silver and hydroxide ions that react to form a precipitate of silver (I) oxide, which can further form the diamine silver (I) ion $2[Ag(NH_3)_2]^+$ known as silver mirror. The reaction of this silver mirror can be seen clearly from the equations below:

$$2Ag^+(aq) + 2OH^-(aq) \rightleftharpoons Ag_2O(s) + H_2O(l) \quad \textbf{(Equation 1.12)}$$

$$Ag_2O(s) + 4NH_3(aq) + H_2O(l) \rightleftharpoons 2[Ag(NH_3)_2]^+(aq) + 2OH^-(aq) \quad \textbf{(Equation 1.13)}$$

1.11.1.2 Silver substrates synthesised via hydroxylamine reduction

Reports have shown that cocaine can be detected in OF at concentrations of 20-50 ng/mL using silver colloids (silver NPs in solution), which are the most commonly used substrates in SERS analysis (Farquharson et al. 2011; Dana et al. 2015).

There are numerous methods for the synthesis of these substrates, which differ in the time of reaction, temperature, reducing agent, aggregating agent and stabilizers. From these, the synthesis of silver NPs by the method of hydroxylamine hydrochloride reduction (using sodium chloride NaCl as an agglomeration agent) has proven to offer NPs with higher SERS activity than other types of synthetic pathways and aggregation agents (Feng 2015). This method also offers the advantage of producing silver NPs at room temperature and under atmospheric conditions. The hydroxylamine reduction method has been proved to generate small mono-dispersive particles with high SERS activity and it has previously been used in the analysis of proteins in OF (Feng 2015).

1.11.1.3 Bimetallic substrates synthesised via seed growth

Core-shell bimetallic silver and gold (Ag-Au) particles have been used for SERS analysis because of their composition, size and unique optical properties involving surface plasmon resonance (Mohammad et al. 2010, Wang et al. 2015). Wang et al. (2015) reported the detection of a small molecule Rhodamine B (MW: 479) at concentrations as low as 2 ng/mL in food products, using a portable Raman spectrophotometer and optimised Au-core/Ag-shell NPs. As the authors stated, the optimised Au-core/Ag-shell NPs could be used as a sensitive SERS substrate to detect trace species (Wang et al. 2015). Thus, offering the possibility of detecting drugs such as cocaine in OF and oral tissue at biological concentrations.

The synthesis of this substrate involved the use of IP_6, which is a non-toxic reagent that can chelate with metal ions to form stable NPs. Initially, gold seeds are formed by the interaction of inositol hexaphosphate (IP_6) with gold ions, then silver ions are added to the gold seeds to create a homogeneous cover over the core of the gold seed. The mechanism under which the Ag-Au core-shell is obtained has not yet been described (Wang et al. 2015).

1.11.1.4 Magnetic Substrates

Metallic NPs can be obtained by diverse number of methods, such as those described above, where particle size and electric properties can be controlled. Aggregation of these NPs is essential to produce hot-spots and therefore gain SERS activity. Most methods use aggregation agents (chemical methods) to control the aggregation of the NPS. Nonetheless, there are reports on the synthesis of paramagnetic metallic NPs where aggregation can be controlled by the use of a magnet (Yang et al. 2015).

Applications of this magnetic NPs as SERS substrate include the analysis of trace pesticide residues at femtomolar levels using portable Raman spectrometry and the detection of drugs (cotinine and benzoylecgonine) from saliva and fingerprints at LOD of 18 ng/mL for cotinine and 29 ng/mL for BZE (Yang et al. 2014, 2015). The detection of low concentrations of analytes using magnetic NPs suggested that this NPs could be used for the detection of cocaine from OF and tongue tissue.

The synthesis of these magnetic NPs was described by Yang et al. (2014; 2015) and is shown in Figure 1.10. Initially, the iron (III) oxide (Fe_3O_4) network is prepared by co-precipitating the oxide with the help of IP_6; then the gold NPs are prepared by the reduction of chloroauric acid ($HAuCl_4$) with sodium citrate. The resulting structure of the substrate is attributed to the presence of the IP_6 and its role of binding and capturing the two different NPs.

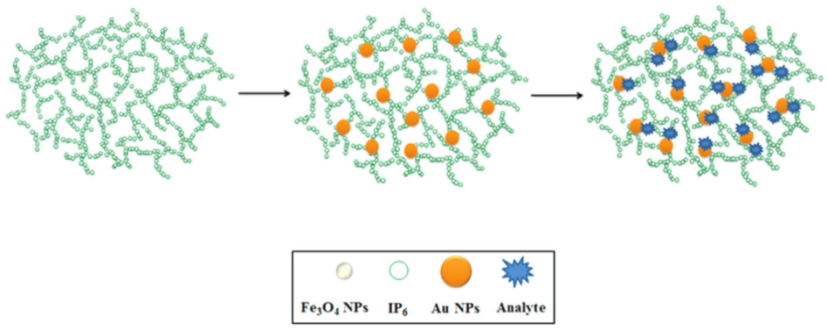


Figure 1.10 Diagram of the fabrication process of S1, from Yang et al. (2014, p.1327). © Wiley-VCH Verlag GmbH & Co. KGaA.

1.11.1.5 Paper based SERS substrates

Paper based SERS substrates have been prepared by different methods, including thermal inkjet printer and dripping (Fierro-Mercado et al. 2012, He et al. 2017). These methods involved the synthesis of gold NPs via chemical reduction (Lee and Meisel 1982). These substrates have the advantage of flexibility, conformability, efficient uptake and absorption of liquid media on the surface of the substrate (Nguyen et al. 2016,

Mosier-Boss 2017). This type of inkjet printing substrates do not need to be prepared in bulk and can be stored for a prolonged period of time (Yu and White 2013). Paper based SERS substrates (inject method) have also been described as substrates that can be obtained in almost any environment with easy fabrication procedures which reduce the cost of the substrate. The advantages that this substrate offers makes it a good candidate for its use in drug testing as the cost of analysis could be reduced significantly compared with other SERS substrates.

Applications of paper-based SERS substrates include the detection of methotrexate (MTX) in human serum and buffered solution of bovine serum albumin (BSA-PBS) at therapeutic levels (of 1.0×10^{-7} M to 3.0×10^{-4} M). The results of these studies showed good linearity when using BSA-PBS ($R^2 = 0.95 \pm 0.02$) and poor linearity when analysing human serum ($R^2 = 0.57 \pm 0.08$) (Fornasaro et al. 2016, Jaworska et al. 2016). Similarly, heroine was successfully detected using paper substrates from solutions of IR780 dye (solution in acetonitrile). The results of this study revealed LOD of 25 ng of heroin on 0.5 μg of IR780 (Yu and White 2013). The LOD reported on these applications suggested that the analysis of cocaine in SOF could be achieved using this type of substrates.

1.11.1.6 Silica-based SERS substrate

Most of the methods that have been described for the preparation of silica-based SERS substrates involve the depositions of NPs by different techniques: (1) Nanolithography, (2) metal film over nanostructures fabrications, (3) sputtering and (4) Laser ablation (Han et al. 2009, Fierro-Mercado et al. 2012, Nguyen et al. 2016). Silica-based substrates offer advantages such as chemical stability, excellent adherence, high enhancement factors, signal homogeneity, ruggedness, simplicity in preparation and uniform nanostructures that are highly reproducible (Nguyen et al. 2016, Mosier-Boss 2017). Immobilization of

silver or gold NPs on the surface of the silica particles results in controlled aggregation of the NPs which produces large enhancements in the Raman signal of adsorbed molecules. However, the time consumed in the fabrication of this kind of substrates is long and skilled procedures are required, making this substrate not cost effective.

Applications on the use of silica-based substrates have been reported for the detection and quantitation of anticancer drugs (sunitinib, paclitaxel, irinotecan and a metabolite of irinotecan SN-38) and cocaine at therapeutic concentrations. Detection of cocaine in distilled water was obtained at LOD of 50 ng/mL. The three drugs (Sunitinib, irinotecan and SN-38) were detected at LOD of 18-26, 60-70 and 20-50 ng/mL respectively. Linearity was evaluated at a range of 10^2-10^3 ng/mL paclitaxel (a metabolite of irinotecan), and doxorubicin. Other applications have reported the detection of anthracene and pyrene at LODs of 1.4 and 8 ng/mL, respectively (Mosier-Boss 2017). Melamine (adulterant added to food products to increase their apparent protein content) was detected at LOD of 1 ng/mL (Wang et al. 2014). Similarly to paper base, the silica base substrates suggested that the analysis of cocaine in SOF and tissue could be achieved using this type of substrates.

1.11.2 Raman Instrumentation

The characteristics of the Raman instrumentation plays an important role in the sensitivity of the method (Kiselev et al. 2016). During Raman and SERS analysis the sample is exposed to an excitation source, this source should be able to efficiently excite the molecule of study and the metallic NPs of the substrate. It has been theoretically reported that a maximum enhancement occurs when the laser is tuned to the peak of the plasmon resonance of the substrate (Sharma et al. 2012, Schlücker 2014). However, experimentally it has been shown that maximum enhancement factors are found when the

laser wavelength is shifted to lower wavelengths (blue wavelength of the plasmon resonance) on both the excitation and emission parts of the Raman process. The maximum signal is therefore found when the plasmon frequency is tuned to be slightly red-shifted from the laser wavelength (Sharma et al. 2012).

Lasers are the excitation source in Raman instrumentation (Koljenović et al. 2007). Lasers beams are highly monochromatic, usually with a small diameter that can be further reduced by using lens systems to focus on small samples (~1 mm^3). In the case of Raman microscopy the diameter of the beam can be as small as 2 μm (Turrell and Corset 1996). The power of the laser can be controlled by the use of different type of laser such as gas lasers (Argon Ar^+, Krypton Kr^+, Helium-Neon He-Ne) or solid-state lasers such as Neodymium –YAG (1064 nm). Diode lasers can be obtained at a specific wavelength in the blue or the infrared regions (Turrell and Corset 1996, Thomson 2002, Gnyba et al. 2011, Trapping and Sorting 2015).

In the current Raman spectrometers, the detection of the Raman signal is conducted by Charge Coupled Devices (CCDs) (Ali and Edwards 2010). The development of compact, low-power laser sources, optical components have resulted in the manufacture of handheld and portable Raman spectrometers that are now available from several manufacturers. This new developments allow the measurement of sample in field (*in-situ*) for real-time chemical detection (Manoharan et al. 1996). Furthermore, stand-off detection by SERS can be possible with Raman microscopes and optical fibre probes.

1.12 THESIS RATIONALE

The initial concept of drug transport or release into OF was based on a philosophy that drugs present in the blood could pass through the cell membranes of the salivary glands across a concentration gradient, the extent of which would be determined by the drug's lipid solubility and pKa value. Whilst this theory holds for many medicinal drugs orally administered, some authors have demonstrated that for cocaine, which is commonly consumed via nasal insufflation or smoking, the S/P ratios are higher than 1.0 and detection times are significantly longer than would be expected using pharmacokinetic models (Osselton et al. 2001, Huestis and Cone 2004). This lead to the proposition that cocaine could form depots in the mouth tissues following exposure and could subsequently be released from the tissue over time, thus increasing the concentration of cocaine in OF and its time of detection.

This research investigated cocaine absorption and excretion from oral/buccal tissues by the use of a modified human kinetic study (i*n vivo* study) and *in vitro* release studies to evaluate the impact of drug depots in the concentration of drugs in OF. Previous i*n vivo* studies demonstrated that following ingestion of coca tea, cocaine and BZE were detected in OF at concentration above the cut-off level (8 ng/mL) (EWDTS 2015) for up to one hour (Reichardt 2014). However, release profiles and windows of detection were not evaluated in the study. Reichardt's study also revealed a number of unexplained artefacts relating to the random detection of cocaine derivatives (AEME, EME, NC and CE) in OF collected after the consumption of coca tea. The analysis of OF samples containing cocaine and cocaine derivatives collected over a prolonged period of time (four hours) following ingestion or swirling of a cup of coca tea could allow the determination of detection windows as well as release profiles using an *in vivo* safe model. Additionally, it could help to understand the unexpected artefacts reported by Reichardt (2014) and

confirm the presence of other cocaine derivatives in OF following the consumption of coca tea. Furthermore, any differences in the release of cocaine/cocaine derivatives (cocaine metabolites and related products such as AEME) from ingestion of coca tea and exposure of the tea to the oral cavity could help evaluate the impact of the contamination of the oral cavity and the release of drugs from drug depots in OF drug testing.

The use of coca tea in *in vivo* studies allowed the evaluation of the release of cocaine and cocaine derivatives into OF safely and with reduced ethical considerations. Coca tea contains small quantities of cocaine derivatives such that at high altitude it eases breathing and reduces the symptoms of altitude sickness. Coca tea does not produce any form of "high" or intoxication and is significantly less likely to affect an individual than the social use of alcohol (Jenkins et al. 1996).

In order to confirm the results of the *in vivo* study and evaluate the kinetics of release of cocaine and cocaine derivatives a modified *in vitro* model using Franz diffusion cells was used. This was achieved by measuring the release of cocaine and AEME into synthetic oral fluid (SOF) across porcine buccal mucosa. By mimicking the *in vivo* process, where drugs such as cocaine are initially absorbed into the oral mucosa following oral exposure (dose) and then released into OF, it is possible to monitor the release profile as well as the permeability of the buccal mucosa. The use of *in vitro* permeability or diffusion studies has been widely used to determine the barrier nature as well as evaluating the kinetics of the tissue and/or the drug in use (Nicolazzo and Finnin 2008). *In vitro* models are commonly used instead of *in vivo* models because of the reduced cost and because they offer comparative diffusion results (Nicolazzo and Finnin 2008, Castro et al. 2016).

The evaluation of the kinetics of cocaine and cocaine derivatives in oral tissue using the modified *in vitro* model could contribute to the understanding of the transport of cocaine from drug depots formed in oral tissues into OF. Furthermore, it could contribute to the kinetics of cocaine across oral mucosa as only few authors have reported the accumulation and permeability of cocaine into tissues with non-keratinised epithelia such as nasal or epithelial cell models (Bhat et al. 2001, Zhang et al. 2012, Clemons et al. 2014). Zhang et al. (2012) reported that the transport across nasal mucosa (ranged 0.2-1.0 μg/min/cm^2) was similar to the olfactory mucosa (range 0.2-0.9 μg/min/cm^2) for concentrations ranging 1-5 mM and that permeability across these tissues was dose-dependent.

Since the high percentage of tissue in the oral cavity is composed of lining mucosa and drugs are more permeable through the non-keratinised epithelium, it was evident that buccal (cheek) mucosa should be used for the development of *in vitro* diffusion studies. However, confirmation of the presence of drug depots has only been reported on porcine tongue tissue (Reichardt 2014). Because of the physiology of the dorsum of the tongue, i.e. keratinised epithelium and decreased permeability compared with the lining mucosa, it can be assumed that formation of drug depots is limited in the dorsum of the tongue than in the lining mucosa. From the lining mucosa, the buccal mucosa offers a relatively immobile surface for ensuring controlled release systems (Madhav et al. 2009) and drug depots could be more likely to be formed in this tissue. While the sublingual tissue (part of the lining mucosa) has non-keratinised epithelium and its high permeability allows rapid transport of drugs into the systemic circulation, its high blood flow, constant saliva washing and tongue activity, could make difficult for drugs to reside in this tissue. In these studies, SOF was used instead of neat (authentic) OF because of the large volumes

of neat OF required. In research, SOF is commonly used to overcome limitations of the low volume of neat OF required in *in vitro* studies (Anizan et al. 2015).

In order to monitor the release of cocaine from oral tissues into SOF in real time, Raman spectroscopy was evaluated. Raman spectroscopy and more specifically SERS, could offer the possibility of detecting drugs in both OF and tissue in a shorter period of time with minimal to no sample pre-treatment and at concentrations below accepted cut-off levels (cocaine cut-off 8 ng/mL in OF) (EWDTS 2015). Although this technique has been used in therapeutic drug monitoring, its implementation in OF drug testing is still novel. Studies on the application of Raman spectroscopy could contribute to its importance as an alternative technique for OF drug testing, as its use could significantly reduce the cost and time of analysis. Detection and quantification of drugs in OF and tissue are commonly conducted by conventional techniques such as LC-MS. However, these techniques are time-consuming and monitoring of the release of drugs from oral tissue into OF cannot be achieved in real time.

1.13 AIM AND OBJECTIVES

1.13.1 AIMS

This research aimed to enhance our knowledge on the absorption and subsequent release of drugs from oral drug depots into oral fluid and to contribute to the interpretation of drug concentration in oral fluid. It also aimed to evaluate alternative techniques for the detection of drugs in oral fluid and oral tissues.

The study proposed to test the following hypotheses:

1. Raman spectroscopy could be implemented in the detection and quantification of cocaine in OF and oral tissue and allow the monitoring of the release of drugs from drug depots in oral tissue into oral fluid in real time.

2. Oral exposure to coca tea could aid in the understanding of oral contamination and elimination of cocaine and its metabolites from formed oral drug depots into oral fluid, on an ethically *in vivo* safe model.

3. I*n vitro* diffusion studies could be used to determine the kinetics of release of drugs from drug depots in oral tissues.

1.13.2 OBJECTIVES

The proposed objectives of this research are related to the measurement and understanding of the phenomenon of the release of drugs from drug depots in oral tissues and their effect on the analysis of drugs from oral fluid.

1. To evaluate the use of Surface Enhancement Raman Spectroscopy (SERS) in the detection and quantification of cocaine in synthetic oral fluid and porcine oral tissue using homemade substrates.

2. To assess the use of portable Raman spectroscopy and Raman microscopy for the detection and quantification of cocaine in synthetic oral fluid and porcine oral tissue.

3. To develop and validate LC-MS quantitative methods for the analysis of cocaine and derivatives in oral fluid, synthetic oral fluid and porcine oral tissue.

4. To evaluate the stability of cocaine and derivatives in buffered oral fluid, synthetic oral fluid and porcine oral tissue under different storage conditions.

5. To investigate the elimination profile and time of detection window of cocaine and its metabolites in collected oral fluid samples following ingestion or swirling of a cup of coca tea.

6. To identify the factors involved in the release of drugs (cocaine and anhydroecgonine methyl ester) from oral tissues using and *in vitro* model that could measure the release of drugs from drug depots into oral fluid.

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Chapter 2

EVALUATION OF HOMEMADE SERS SUBSTRATES AND HANDHELD RAMAN SPECTROSCOPY FOR THE ANALYSIS OF COCAINE IN SYNTHETIC ORAL FLUID AND PORCINE ORAL TISSUE

2.1 INTRODUCTION

Detection and quantification of drugs in oral fluid (OF) and tissues are commonly conducted by analytical techniques such as gas/liquid chromatography couplet to mass spectrometry (GC-MS or LC-MS). These techniques, however, are time-consuming as sample preparation procedures are generally required to increase the sensitivity and robustness of the GC/LC-MS method. In comparison, Raman spectroscopy offers the possibility to detect drugs in both OF and tissue in a shorter period of time (few minutes) with minimal to no sample pre-treatment (Section 1.11). Furthermore, Surface Enhanced Raman Spectroscopy (SERS) may have a potential to allow the detection of drugs such as cocaine at physiological concentrations.

In human OF samples, cocaine can be detected at concentrations as high as 3 μg/mL following smoke of crack-cocaine (Kato et al. 1993; Cone 2012) and up to 9 μg/mL following immediate drinking of a cup of coca tea (Reichardt 2014). In *in vitro* studies cocaine was detected in synthetic oral fluid (SOF) at concentrations of 0.42-1.3 μg/mL and at 0.08-0.87 μg/mL in tissue homogenates following exposure to the smoke of 200 mg crack-cocaine (Reichardt 2014). Although cocaine can be detected at concentrations of up to 9 μg/mL in human OF samples and up to 1.3 μg/mL in SOF from *in vitro* studies, any OF samples at concentration above 8 ng/mL would also give a positive result (EWDTS 2015).

After complexation with SERS substrate, drugs were detected in biological and non-biological samples at concentrations in the range of 2 ng/mL to 10 μg/mL (Farquharson et al. 2011; Dana et al. 2015; Feng 2015). Which implies that cocaine could be detected in OF at concentrations below the cut off concentration of 8 ng/mL. The substrates used

in the studies that allowed the detection of drugs at concentrations below the cut-off concentration for cocaine were silver, gold or a mixture of silver and gold nanoparticles (NPs) obtained by the following procedures: the formation of diamine silver ions, reduction of silver nitrate to silver ions or seed growth of silver and/or gold NPs. Furthermore, the substrates used were applied on liquid and solid samples and therefore had the potential to be used for the analysis of drugs in SOF and porcine tongue tissue samples.

Wrona et al. reported that diamine silver ions formed via Tollens reagent (Equation 1.13) were used in the analysis of analytes in liquid and solid state such as edible oils (2015) and plastics (2017). Similarly, silver NPs synthesised by the method of hydroxylamine hydrochloride reduction (Feng 2015) produce a liquid substrate that could be applied on SOF and tissue samples. This synthesis has proven to generate small mono-dispersive NPs that can be used to detect cocaine in OF at concentrations of 20-50 ng/mL (Farquharson et al. 2011; Dana et al. 2015). Core-shell bimetallic silver and gold (Ag-Au) particles and magnetic NPs could also be used to enhance the Raman signal for the detection of cocaine in OF and tissue. Limits of detection (LOD) of 2 ng/mL for Rhodamine B (MW: 479) in food products were reported using the Ag-Au particles and a portable Raman spectrophotometer (Wang et al. 2015). Magnetic NPs were used for the analysis of cotinine and benzoylecgonine from saliva and fingerprints at LOD of 18 ng/mL for cotinine and 29 ng/mL for benzoylecgonine (BZE) (Yang et al. 2014, 2015).

In this chapter, Raman spectroscopy and more specifically SERS was evaluated as an alternative technique to LC-MS for the analysis of cocaine in SOF and porcine tongue tissue. Raman analysis could confirm the formation of drug depots in porcine tongue tissue and furthermore could speed the process of the monitoring of the release of drugs

from drug depots into OF. In order to evaluate the SERS technique four different substrates with different morphologies were synthesised and characterised. Substrate characterisation was essential to determine the activity of the substrates. Although in this work there was no intent to develop the plasmonic properties of the SERS substrates, this study faced the challenge of producing sensitive SERS substrates that could be applied to both liquid and solid biological samples for the detection of cocaine at physiological concentrations.

2.1.1 AIM AND OBJECTIVES

2.1.1.1 *Aim:*

The aim of this chapter was to evaluate the use of Raman spectroscopy in the detection and quantification of cocaine in OF and oral tissue using substrates synthesised in the laboratory, i.e. homemade substrates.

2.1.1.2 *Objectives:*

- Synthesise SERS substrates that can enhance the Raman signal of cocaine for the detection of cocaine in synthetic oral fluid and porcine tongue tissue.
- Characterise the synthesised substrates in order to determine their SERS properties.
- Evaluate the synthesised substrates for the detection and quantification of cocaine in synthetic oral fluid and porcine tongue tissue using handheld Raman spectroscopy.

2.2 METHODS

2.2.1 Materials

Crack cocaine was provided by TICTAC Communications, St Georges Medical School, University of London.

Porcine tongues were purchased from The Village Butcher, Kingsclere. Porcine tongue tissue was used due to its physiological and anatomical similarities to human tongue tissue (Simon and Maibach 2000).

Ascorbic acid ($C_6H_8O_6$), ammonia solution (35%), clearene, chloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$), cocaine hydrochloride, ethanol analytical grade, hydroxylamine hydrochloride ($HONH_2 \cdot HCl$), inositol hexaphosphate (IP_6), iron (II) chloride ($FeCl_2$), iron (III) chloride ($FeCl_3$), silver nitrate ($AgNO_3$), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium-potassium tartrate tetrahydrate ($KNaC_4H_4O_6 \cdot 4H_2O$), tri-sodium citrate ($Na_3C_8H_2O_7$), were purchased from Sigma Aldrich (Dorset, UK). Clearene solvent was purchased from Leica Biosystems (Munich, Germany).

2.2.2 Instrumentation

Ultraviolet-visible (UV-Vis) measurements were performed using a Varian Cary 50 Probe UV-Vis spectrophotometer with a xenon flash lamp and dual silicon diode detector. The instrument was run with the Scan application of CaryWinUV software and set up to measure absorbance in dual beam mode, with a range of 200 - 800 nm. The analysis was conducted by the researcher at Bournemouth University.

Scanning Electron Microscopy (SEM) images were obtained using a JEOL scanning electron microscope; model JSM-6010 Plus/LV with InTouchScope software. Gold coating was obtained using a Quorum Q150R ES Sputter Coater. The analysis was conducted by the researcher at Bournemouth University.

Transmission Electron Microscopy (TEM) images were obtained using a JEOL transmission electron microscope; model JEM 1400Plus. Images were recorded and processed using a Gatan Orius camera and Gatan Microscopy Suite Digital Micrograph Software version 2.11.1404.0. The analysis was conducted by the researcher in collaboration with Hospital Fundación Santa Fe de Bogotá (Bogotá – Colombia).

Sectioning of tissue at thickness 2-5 mm was conducted using a Brunel bench microtome and a section razor (Brunel microscopes Ltd., Chippenham – UK). Thickness of 0.02 mm were obtained using a Brunel YD rotary microtome (Brunel microscopes Ltd., Chippenham – UK) at an angle of 35°.

Raman measurements were performed using the Rigaku First Guard handheld Raman spectrometer equipped with 532 nm laser power, 60 mW laser output power and charge coupled device (CCD) detector. Raman spectra were collected over the wavenumber range of 250-3000 cm^{-1} and spectral resolution of 10 cm^{-1}. Each spectrum was the sum of three scans, such that each scan was exposed for 14 seconds. The analysis was conducted by the researcher at Bournemouth University.

2.2.3 Synthesis of SERS substrates

In order to develop the SERS analysis, four different substrates were synthesised for their use in the detection of cocaine in SOF and tongue tissue. Substrates consisted of silver and/or gold colloids (Table 2.1). The substrates were not commercially available.

Table 2.1 Details of gold and silver substrates.

Substrate	Substrate Name	Substrate base	Synthesis Method	Reference
S1	Deposition	Silver	CD	(Wrona et al. 2015)
S2	Reduction	Silver	CR	(Feng 2015)
S3	Silver-Gold	Silver and gold	CR	(Wang et al. 2015)
S4	Magnetic	Silver	CR	(Yang et al. 2015)

CD: Chemical deposition, CR: Chemical reduction, S1: Substrate 1, S2: Substrate 2, S3: Substrate 3hemical deposition, S4: Substrate 4.

2.2.3.1 *Synthesis of Substrate 1*

Substrate 1 (S1) was made based on the methodology described by Wrona et al. (2015). The synthesis involved the chemical reduction of an aqueous solution of silver nitrate using a reducing agent of sodium-potassium tartrate in alkaline conditions (pH 12). S1 was generated by deposition of silver nanoparticles (NPs) on a glass surface after mixing Solutions 1 and 2 (see below) in a 1:1 ratio. The silver NPs were deposited on cover glasses (Borosilicate glass, 22x40 mm) that were previously washed with ethanol and dried under a stream of nitrogen.

Solution 1: Ammonia solution (35%) was added drop by drop to a 14 mL silver nitrate solution (62 mg/mL), the initial addition of ammonia solution produced a chocolate-coloured precipitate that then disappeared. An extra 1 mL of silver nitrate solution was

added to obtain a turbid solution. The final solution was diluted to a final volume of 100 mL with ultrapure water and stored in an amber glass bottle to be protected from light.

Solution 2: Potassium sodium tartrate (0.19 g) was added to 100 mL of boiling ultrapure water. Then 20 mL of aqueous silver nitrate (110 mg/mL) was slowly added with vigorous stirring using a magnetic stirrer. The solution was left to boil for 10 min and then allowed to cool on standing at room temperature. The solution obtained was filtered with a Millex-HA Millipore filter (membrane diameter: 33 mm, pore size: 0.45 μm) and kept protected from light.

2.2.3.2 *Synthesis of Substrate 2*

Substrate 2 (S2) was prepared based on the method described by Feng et al. (2015). The silver NPs were obtained by the reduction of silver nitrate by hydroxylamine hydrochloride. Initially, 9 mL of 0.1M sodium hydroxide was added to 10 mL of 0.06M hydroxylamine hydrochloride to adjust the hydroxylamine solution at pH of 12. This solution was then rapidly added to a 180 mL of 0.1mM silver nitrate. The appearance of the resulting solution was milky-grey. The final solution was stored in an amber glass bottle.

Before SERS analysis, the colloidal solution (200 mL) was centrifuged for 30 min at 10,000 rpm and 140 mL of the supernatant was eliminated to concentrate the substrate. Aqueous 0.4M sodium chloride (1.2 mL) was added to the substrate (final concentration of 8mM) to aggregate the NPs.

2.2.3.3 *Synthesis of Substrate 3*

Substrate 3 (S3) was prepared based on the method described by Wang et al. (2015). To obtain the core-shell NPs (S3), an initial 5 mL of 0.001M IP_6 was added to 100 mL of 0.025mM chloroauric acid and then heated to boiling using a hot plate. Then 1 mL of 0.04M tri-sodium citrate was added at a rate of 0.1 mL/min while stirring vigorously using a magnetic stirrer to obtain a solution of iron oxide (Fe_3O_4) NPs. A mixture of 80 mL of 1.3mM silver nitrate solution and 1 mL of the previously prepared Fe_3O_4 solution were then mixed while stirring at room temperature. Then 5 mL of 0.1M acetic acid was added drop wise while stirring. The substrate was obtained after 10 min.

2.2.3.4 *Synthesis of Substrate 4*

The magnetic Substrate 4 (S4) was prepared based on the method described by Yang et al. (2014). The fabrication procedure of S4 involved two steps, which included the preparation of the iron (III) oxide (Fe_3O_4) network and the preparation of gold NPs by the reduction of chloroauric acid ($HAuCl_4$) with sodium citrate. S4 was synthesised at atmospheric pressure.

Preparation of the Fe_3O_4 network: Solution 1 was obtained by adding 5 mL of 0.001M IP_6 to 150 mL of ultrapure water. This solution was heated to boiling with vigorous stirring using a hot plate and a magnetic stirrer. Solution 2 was obtained by mixing 0.318 g of iron (III) chloride and 0.130 g of iron (II) chloride in ultrapure water. 5 mL of Solution 2 was added to the boiling Solution 1 to obtain Solution 3 and then allowed to stir for one hour. After 1 hour of stirring, 1.2 mL of 0.4 M sodium hydroxide was added drop by drop into Solution 3 to obtain Solution 4. After 30 minutes of stirring

5 mL of 0.001M IP_6 was added to Solution 4 and the final solution was stirred for another 30 minutes. The final solution contained black-brown magnetic NPs (Fe_3O_4 network) that were collected using a magnet. The Fe_3O_4 network was rinsed with ultrapure water seven times to remove the excess of reagents.

Addition of gold NPs to the Fe_3O_4 network: The magnetic Fe_3O_4 network was dispersed in 150 mL of ultrapure water and then heated to boiling with strong stirring. Then 5 mL of chloroauric acid 0.03 M was added and the solution was refluxed for 15 minutes. After 15 minutes of refluxing, 10 mL of sodium citrate 0.04 M was added rapidly into the boiling solution and refluxed for 45 minutes. The colour of the mixture changed from black-brown to reddish brown. The reaction mixture was allowed to cool on standing at room temperature and the resulting NPs (S4) were collected by a magnet and washed seven times with ultrapure water.

2.2.4 Substrate Characterisation

Characterisation of NPs with SERS properties can be achieved by measuring the resonance absorbance of the plasmoid in the visible region of the absorption spectra, which is obtained by UV-Vis spectrometry (Cañamares et al. 2005). Characterisation by this technique was only conducted for S2, S3 and S4. S1 could not be characterised by UV-Vis because of its solid state. Additionally, the morphology of the NPs was obtained by electron microscopy (Table 2.2) using SEM and TEM techniques, which are the most common techniques used for the measurement of particle size and plasmon identification (Desai et al. 2012, Williams 2015).

Table 2.2 Substrates characterisation methods.

Substrate	Name of substrate	Characterisation technique
S1	Deposition	SEM
S2	Reduction	UV-Vis, TEM
S3	Silver-Gold	UV-Vis, TEM
S4	Magnetic	UV-Vis, TEM

S1: Substrate 1, S2: Substrate 2, S3: Substrate 3, S4: Substrate 4, SEM: Scanning electron microscopy, TEM: Transmission electron microscopy, UV-Vis: Ultraviolet-Visible.

2.2.4.1 *UV-Vis spectroscopy*

UV-Vis spectra of S3, S3 and S4 were obtained by diluting 300 µL of the substrate in ultrapure water to a final volume of 1 mL. Samples were measured using disposable semi-micro 1.5 mL polymethyl methacrylate (PMMA) Cuvettes at room temperature. Spectra of S1 could not be obtained because of its solid state. UV-Vis spectra were collected on day 0, 3, 7 and 19 in order to monitor the particle size of NPs. An increase in particle size was an indication of poor stability (Larmour et al. 2012).

2.2.4.2 *Scanning Electron Microscopy (SEM)*

S1 was initially gold coated to a final thickness of 5nm. SEM images were then obtained with a JEOL scanning electron microscope at 20 kV using the option of Secondary Electron in high vacuum mode. Particle size measurements and count were acquired using ImageJ software version d 1.47.

2.2.4.3 *Transmission Electron Microscopy (TEM)*

S2, S3 and S4 were initially washed seven times with ultra-pure water to clean the NPs and eliminate any excess of reagents left from the synthesis. The NPs were re-suspended in water and subsequently deposited on cupper grids of 400 mesh (Agar Scientific) using a capillary. Samples were protected from light at all times.

TEM images were obtained at different magnifications (range 20,000x – 30,000x) using a JEOL transmission electron microscope. Particle size measurements and count were acquired using ImageJ software version d 1.47.

2.2.5 Synthetic Oral fluid preparation

SOF was prepared using the Cozart Biosciences protocol (2008) "Production of Synthetic Saliva" (Appendix A).

2.2.6 Exposure of porcine tongue to crack-cocaine

Exposure of tongue tissue was conducted following the procedure described by Reichardt (2014). The porcine tongue was suspended in a smoking chamber and exposed to the smoke generated by burning 200 mg crack cocaine (Figure 2.1). The smoking chamber was connected to a vacuum pump that created an airflow of 0.5 m/s. The latter conditions were made in order to achieve sufficient suction to mimic normal smoking conditions. The crack cocaine was heated using a Bunsen burner and exposure to cocaine continued until smoking ceased. After exposure to cocaine smoke, the tongues were washed seven times with SOF to remove any excess of cocaine from the surface of the tongue.

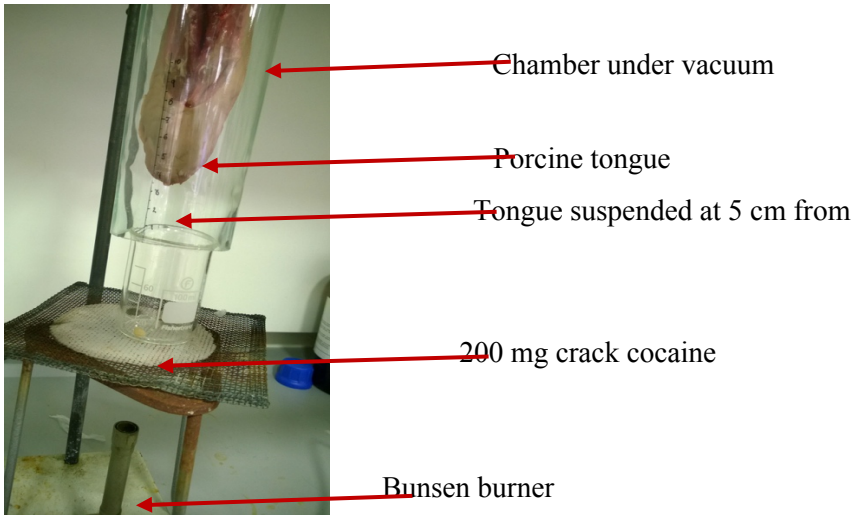


Figure 2.1 Exposure of tongue tissue to 200 mg crack cocaine.

2.2.7 Oral fluid sample preparation

Samples of SOF with and without cocaine (control SOF) were prepared by serial dilutions from a stock solution (10 mg/mL cocaine hydrochloride in ethanol). The final concentrations of cocaine in the SOF samples were 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL.

2.2.8 Tissue sample preparation

Cross sections of control tissue and tissue containing cocaine were cut from 1.5 cm above the tip of the tongue. Tissue sections at 5, 2 and 0.02 mm were obtained by two different cutting methods (bench microtome and rotary microtome cutting respectively) to determine the best working thickness of the sample.

2.2.8.1 *Bench Microtome cutting*

Initially, control and exposed tongue tissue were frozen at -20 °C. A total of 12 samples were subsequently cut from frozen using a Brunel bench microtome and a sectioning razor at thickness of 5 and 2 mm.

2.2.8.2 *Rotary Microtome cutting*

Tissue samples with dimensions of 50 x 50 x 50 mm were fixed in 10% formalin solution for 48 hours and embedded into paraffin wax prior to sectioning. Tissue sections were cut using a Brunel rotary microtome. Tissue sections were floated into a 30°C water bath in order to reduce shrinking and creasing of the tissue. Floated tissues were picked up using microscope slides. The slides were then heated at 37 °C for 48 hours. Finally, tissues were de-waxed using clearene solvent and rehydrated using five solutions with decreasing amounts of alcohol in water (70, 50, 30, 10 and 0 % v/v).

2.2.9 Analysis of Oral Fluid Samples - Acquisition of Raman spectra

Samples of control SOF and SOF containing cocaine were analysed using the Rigaku 532 nm handheld Raman instrument with and without the addition of SERS substrates.

Raman spectra were obtained by directly exposing the samples to the laser beam. SERS spectra were obtained by measuring the scattering of the samples mixed with substrate. Six replicates were collected for each experiment. The optimal number of replicates was calculated based on the intraclass coefficient (ICC) (Saha et al. 2012) of a preliminary data set from 23 samples at three concentrations (200, 500 and 1000 mg/mL cocaine in SOF). The ICC was calculated based on the Equation 2.1. The ICC was >0.90 for all concentrations for 3 and 6 replicates with ICC ranging 0.93 to 0.98. Comparison between the spectra obtained with the matrix (SOF and porcine tissue) and matrix containing cocaine was used to evaluate the presence of cocaine. Cocaine was confirmed by the presence of the peak at 1000 cm^{-1} in all samples.

$$ICC\ of\ mean\ of\ \text{'m' replicates} = \frac{between\ variance}{(between\ variance + \frac{within\ variance}{m})} \quad \textbf{(Equation 2.1)}$$

The between variance is the inherent sample heterogeneity or variability (pooled across the three concentrations) and within variance is the within-sample variability. When ICC is close to 1, the reliability increases and when ICC = 1 there is no sample variability.

2.2.9.1 *Method Optimisation*

The following parameters were evaluated: (1) Concentration of sample required to obtain Raman signal. (2) Optimal amount of sample mixed with the substrate. (3) Optimal time of interaction between the substrate and the samples, i.e. sample/substrate ratio. (4) Concentration range including limit of detection (LOD) of cocaine in SOF and linearity. (5) Accuracy and precision, and (6) matrix effect. Some of the parameters evaluated (accuracy, precision, linearity and range) are part of the criteria recommended for the validation of quantitative Raman methods described by the United States Pharmacopeia (USP 2011). The other parameters (sample to substrate ratio and time of interaction) were evaluated based on different times/ratios observed in the literature. Because of low sensitivity obtained with some of the substrates (S1 and S4), some of the parameters could not been evaluated.

The **sample to substrate ratio** was evaluated on six sets of samples at ratios of 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10 sample to substrate. The **time of interaction** between the sample and the substrate was evaluated in three set of samples at 5, 30 and 60 minutes. The **LOD** was determined as the lowest concentration at which a three-to-one signal-to-noise ratio (S/N) could be obtained. The S/N was based on the peak height from the baseline noise. The **linearity** of the method for cocaine in SOF was evaluated over a concentration range of 0.1-1 mg/mL using six standards. The regression line was

calculated by the method of least squares and expressed by the correlation coefficient (R^2), linearity was assessed by F-test and visual evaluation of residual plots.

Intra-day and inter-day precision and accuracy of the method was determined at a low (0.1 mg/mL), medium (0.4 mg/mL) and high (1 mg/mL) concentration. **Intra-day precision** was calculated using six (n = 6) sets of samples obtained on the same day at each concentration level and expressed as a percentage relative standard deviation (RSD). **Inter-day precision** was evaluated at each concentration level on three different days (n = 3) and expressed as a percentage of RSD. An acceptable value of ±20% of the reference value was used for intra-day and inter day precision (USP 2011). **Accuracy** was calculated by dividing the mean measured concentration at each level (n = 6) by the theoretical spiked concentration and expressed as a percentage of the theoretical spiked concentration. Accuracy was reported as acceptable if the measured concentration was ±20% of the theoretical value. The **matrix effect** which is the effect of SOF constituents in the detection of cocaine was assessed by comparing the Raman intensity of cocaine when SOF had been diluted with water at dilutions factor of 1.5, 2, 3, 4, 6, 9, 17 and 34 (n = 8).

2.2.10 Analysis of Tissue Samples - Acquisition of Raman spectra

SERS analysis using S1, was conducted by depositing the NPs on the surface of the tissue. Analysis using S2-4, was conducted by depositing 200 µL of substrate on the surface of the tissue using a pipette and allowed to dry at room temperature.

2.2.11 Spectral pre-treatment and treatment

All spectra were dark background corrected using the inbuilt Micro 20/20 software from the First Guard Raman instrument. Comparison between spectra was conducted by setting the baseline in the proximities of the cocaine Raman peak (1000 cm^{-1}) to zero. Statistical analysis was conducted using the software ~IBM SPSS Version 23. Kruskal-Wallis Test H test was used for comparison of groups. Results with 2-tailed $p_s < 0.05$ were considered significant.

2.3 RESULTS AND DISCUSSION

2.3.1 Characterisation of SERS substrates

2.3.1.1 *Characterisation of Substrate 1*

The synthesis of S1 resulted in homogeneous depositions on the glass base. The SEM images (Figure 2.2-S1) showed the aggregation of the NPs and indicated that hot-spots could be formed. Hot-spots are regions where the electromagnetic camp is intensified because of the proximity of the plasmonic surfaces.

The morphology and particle size of S1 were similar to those reported in the literature (particle size ranging 80-230 nm) (Wrona et al. 2015, 2017). Spherical particles with distribution sizes between 70-210 nm were seen in the micrographs of the synthesised substrate. Average particle size for S1 was 110 ± 20 nm.

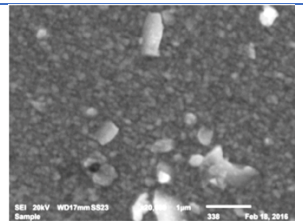
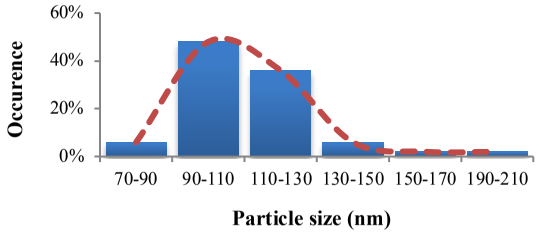
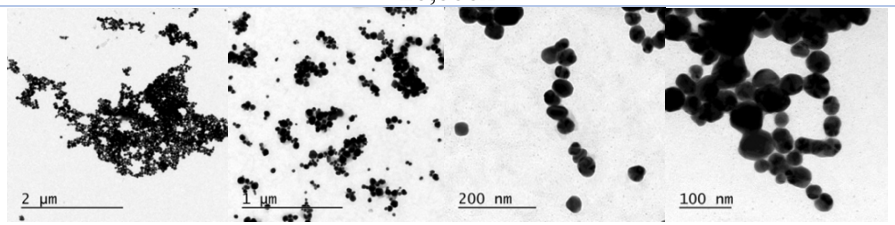
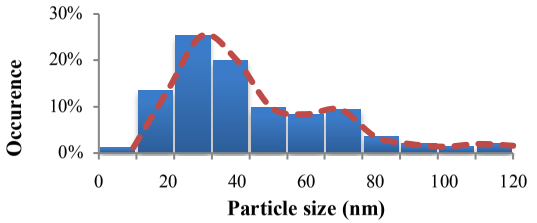
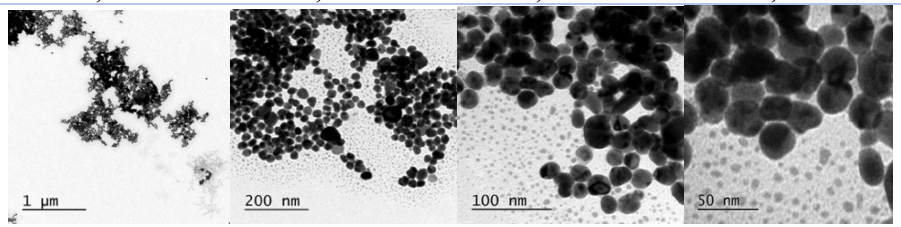
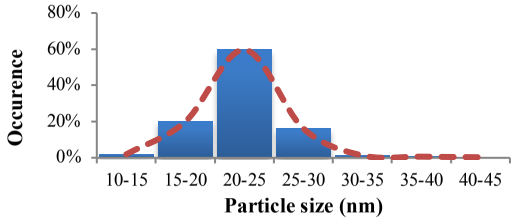
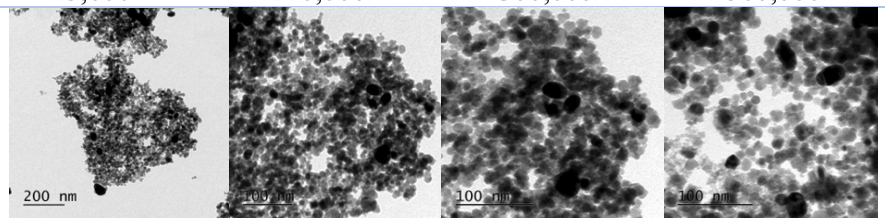
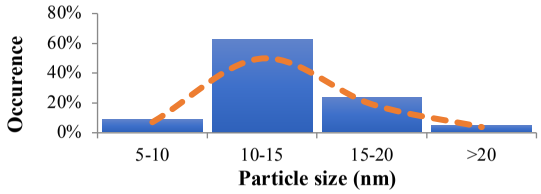
Substrate	Morphology	Particle size distribution
S1	 20,000x	
S2	 20,000x 40,000x 150,000x 200,000x	
S3	 25,000x 120,000x 300,000x 500,000x	
S4	 80,000x 120,000x 250,000x 300,000x	

Figure 2.2 SEM micrograph of S1 (JEOL). TEM micrographs of S2, S3 and S4 (JEM 1400Plus) and size distribution of S1, S2, S3 and S4.

2.3.1.2 *Characterisation of Substrate 2*

Figure 2.3 illustrates the characteristic plamonic resonance at 413 nm obtained for the hydroxylamine substrate S2 using UV-Vis spectrometry. The band at 413 nm indicated the presence of mono-disperse NPs of approximately 30 nm particle size (Agnihotri et al. 2014). A decrease in maximum absorption at longer wavelengths seen in the following days of the synthesis (Figure 2.3) indicated that S1 was conformed of heterogeneous particles with various particle sizes (Mie 1908; Agnihotri et al. 2014). Mie (1908) theory explains, that the position of maximum extinctions is directly related to the size of the NPs and the broadening of the peak (width at half maximum (FWHM) of a peak). Hence, the decrease in UV-Vis band intensity also suggested the possible oxidation of NPs and loss of SERS activity, i.e. the decrease in stability of the substrate.

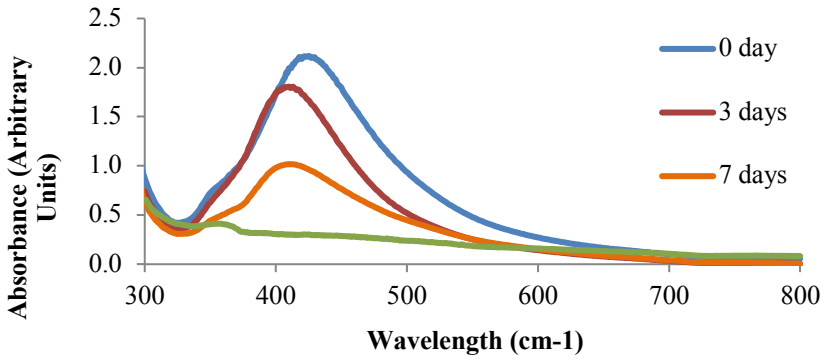


Figure 2.3 UV-Vis spectra (Varian Cary 50) of S2. Spectra collected at times 0, 3, 7 and 19 days.

These results contradicted the results presented by Feng et al. (2015), which stated that the silver NPs obtained using their methodology were stable for up to four months, since the particle size increased and varied with time of storage. The increase and variation in particle size implied that S2 was not stable, therefore this substrate (S2) was synthesised prior to each SERS analysis to ensure the use of small mono-disperse NPs.

Spherical particles with an average size of 50 ± 26 nm and distribution in particle size from 20-120 nm were observed in the TEM micrographs (Figure 2.5-S2). The histogram in Figure 2.7-S2 indicates that there was a maximum percentage of NPs in the range between 30-50 nm. Aggregation of silver NPs were seen in all images, even though the samples were sonicated several times during the TEM sample preparation. This aggregation inferred the possibility of hot-spot formation and therefore the high activity of the substrate.

2.3.1.3 *Characterisation of Substrate 3*

Figure 2.4 illustrates the characteristic plasmonic resonance of S3 at 412 nm. This resonance was more characteristic of silver NPs than gold NPS, which was attributed to the higher amount of silver NPs in the complex. The absorbance of silver NPs has been reported at approximately 400 nm, whereas gold NPs absorb at around 550 nm (Herrera et al. 2013). The silver to gold molar ratio used for the synthesis of this substrate was 36:1. Only one band was present in the UV-Vis spectra indicating that the silver NPs homogeneously covered the core of the gold seed. A small variation (< 0.5%) on the maximum absorption was seen in the following days, which indicated that the S3 was very stable.

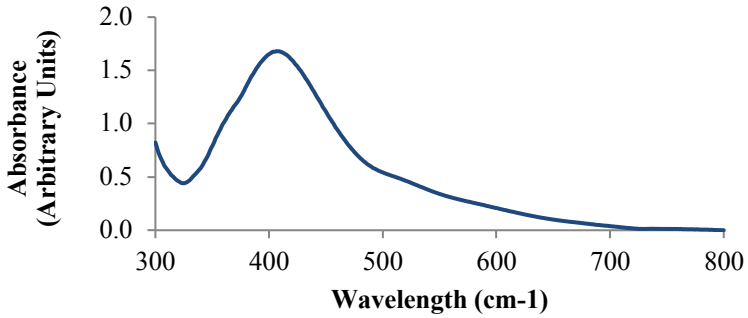


Figure 2.4 UV-Vis spectrum (Varian Cary 50) of S3.

TEM images, on the other hand, showed that S3 was composed of spherical particles with average size of 22 ± 4 nm (Figure 2.2-S3). The histogram illustrated in Figure 2.2-S3 indicated that there was a maximum percentage of NPs in the range between 20–25 nm.

Although all of the particles had a spherical shape, there were marked differences in the colour and characteristics of the NPs. The morphology obtained for S3 was compared with the morphology reported by Wang et al. (2015) to confirm the core-shell morphology. Three primary particle types were observed with a substantial fraction of the NPs having a core-shell structure: (1) Particles with a homogeneous dark colour (thick shell) indicated the inadequate amount of gold seeds used in the synthesis. (2) Particles with empty shells. (3) Particles with core-shell morphology.

Aggregation of Ag-Au NPs was observed in all micrographs, even though samples were sonicated several times during TEM preparation. This aggregation inferred the possibility of hot-spot formation and therefore the high activity of the substrate which could increase the LOD of cocaine in SOF samples.

2.3.1.4 *Characterisation of Substrate 4*

Synthesis of the Fe_3O_4 network was achieved under atmospheric conditions. This solution had a characteristic dark brown colour and the particles were aggregated by a magnet. Figure 2.5 illustrates the solutions' colour and the magnetic response of both the Fe_3O_4 network and the final magnetic S4. These initial results indicated that synthesis of the Fe_3O_4 network was successful. Furthermore, synthesis of the magnetic NPs led to a

dark red coloured solution , characteristic of the magnetic NPs (Yang et al. 2015) with magnetic properties.

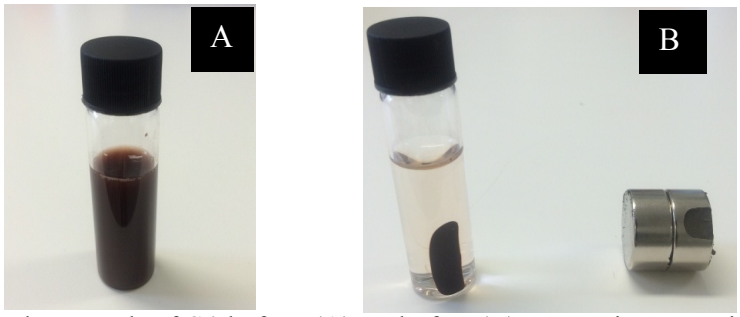


Figure 2.5 Photograph of S4 before (A) and after (B) magnetic separation by an external magnet.

The characterisation by UV-vis for S4 was not conclusive because no characteristic absorption was seen in the spectra (Figure 2.6). These results were attributed to the small percentage (32%) of gold NPs in the substrate.

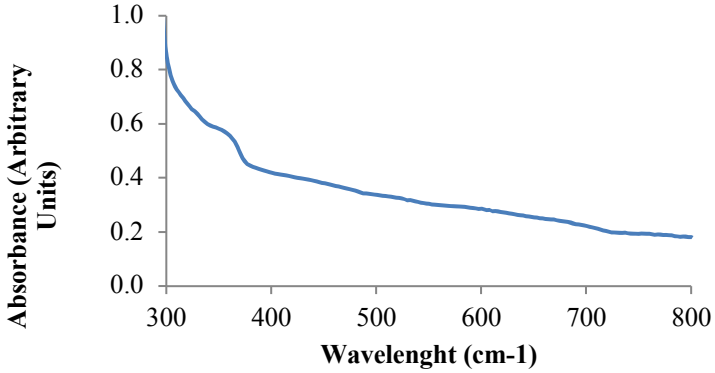


Figure 2.6 UV-Vis spectrum (Varian Cary 50) of S4.

Spherical particles were observed for S4 in the TEM images with mean particle size of 13 ± 3 nm and range of 10-15 nm for the majority of the magnetic NPs (Figure 2.2–S4).

The micrographs showed (1) clear particles (IP_6), (2) clear particles with dark spots (Fe_3O_4) and (3) dark particles (Au) some of which were also embedded into the clear

particles. The morphology of S4 and the morphology reported by Yang et al. (2015) showed similarities in the shape and colour of the NPs. However, a higher amount of gold particles was seen in comparison to the embedded Fe_3O_4. The particle size obtained for the NPs (13 nm) were similar to those reported for the magnetic substrate (10-11 nm) (Yang et al. 2015).

2.3.2 SERS analysis of synthetic oral fluid and tissue samples.

This section presents the SERS results of the detection of cocaine from SOF and tongue tissue samples using the four substrates described previously. Characterisation of the substrates (section 2.2.4) showed that all substrates could have plasmonic properties and therefore be able to enhance the Raman signal of cocaine significantly.

The initial Raman analysis of solutions of cocaine in SOF at a concentration of 10 mg/mL was unsuccessful and no Raman signal was obtained, for this reason, SERS analysis was evaluated. A higher limit of detection of 1 mg/mL cocaine in SOF was set for SERS analysis. This concentration was chosen as 100 times the maximum theoretical concentrations of cocaine in OF that could be obtained from *in vitro* and *in vivo* studies (< 0.008 mg/mL cocaine in OF). Samples at concentrations equal or above 1 mg/mL cocaine in SOF that did not show a visible signal after enhancement with SERS substrates were not further evaluated.

2.3.2.1 *Analysis of synthetic oral fluid and tissue samples using Substrate 1*

None of the SERS spectra of either SOF or tongue tissue using S1 showed any of the characteristic peaks of cocaine (Farquharson et al. 2011, Yang et al. 2015), which

indicated that cocaine could not be detected in these samples using S1 (Figure 2.7). Different volumes of SOF with high concentrations of cocaine (range 1-10 mg/mL) were evaluated unsuccessfully. Farquharson et al. (2011) and Yang et al. (2015) reported Raman scattering at approximately 872, 999, 1026, 1273, 1597 and 1716 cm^{-1} for the presence of cocaine (Farquharson et al. 2011, Yang et al. 2015). Figure 2.7 illustrates the different SERS spectra obtained for S1 (control), S1 mixed with a solution of cocaine in ethanol (10 mg/mL) and S1 deposited on the surface of the tissue.

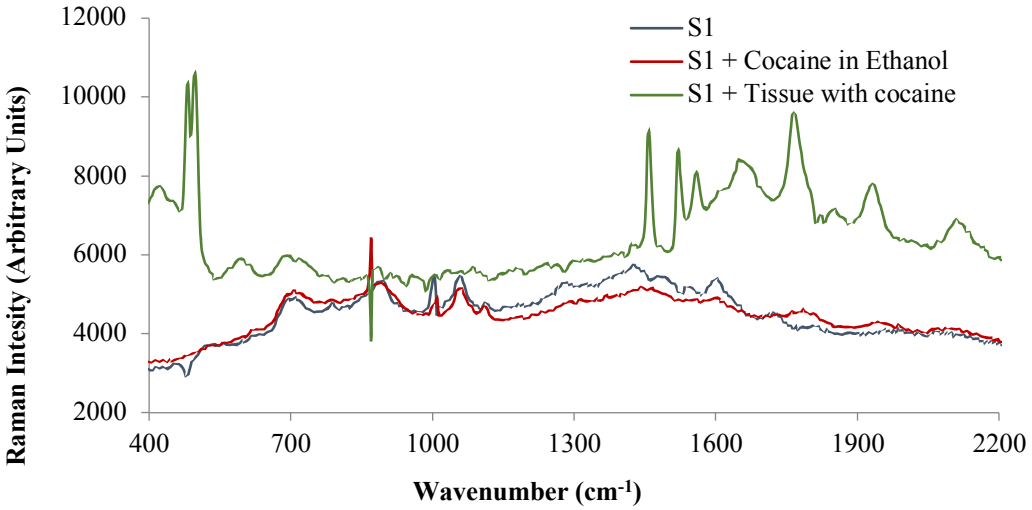


Figure 2.7 SERS spectra (Handheld Raman 532 nm, Rigaku) of Substrate 1 (blue color), Substrate 1 mixed with cocaine in ethanol at 10 mg/mL (Red colour) and S1deposited on the surface of tongue tissue containing cocaine.

Deposition of S1 on the surface of the tissue was very challenging because S1 needed to be synthesised on the surface of the tissue. The deposition procedure led to uneven deposition of particles, which varied between tissue samples. Spectra from tissue containing cocaine showed some scattering at wavenumbers of 1455, 1518, 1752 1915 cm^{-1}. These peaks were assigned to the scattering of proteins (CH_2, C=C) inherent of the tissue. However, no peaks could be assigned to the scattering of cocaine in the tissue. Since cocaine in SOF could not be detected at concentrations below 10 mg/mL, it was assumed that no enhancement would be obtained from tissue samples containing cocaine

using S1. Concentration of cocaine in tissue were reported in the range of 25-870 ng/mL (Reichardt 2014).

2.3.2.2 *Analysis of synthetic oral fluid and tissue samples using Substrate 2*

Raman spectra of control SOF, SOF containing cocaine, methanol, S2 substrate with methanol and S2 with cocaine in ethanol (10 mg/mL) were obtained to determine characteristic peaks from the scattering of these samples.

No peaks were seen when control SOF, SOF containing cocaine and S2 were analysed independently. This indicated that any scattering from the mixture of S2 with solutions of control SOF and SOF containing cocaine would be the result of the scattering of cocaine and/or SOF. Comparison of Raman spectra of the control SOF and cocaine in SOF resulted in evident differences in band positions and intensities (Figure 2.8). The spectrum of the SOF did not show any characteristic peaks for cocaine. The solution of cocaine in SOF, exhibited a predominant band at 1704 cm^{-1} (C=O stretching), which was assigned to the tropine ring stretch, the symmetric and asymmetric phenyl ring breathing modes, the C-phenyl stretch, the trigonal phenyl ring breathing mode, and the ester carbonyl stretch of the phenyl ester ring. The band at 1605 cm^{-1} (C=C amide I) was assigned to proteins present in the SOF from which scattering is enhanced by the presence of the cocaine. The band at 1605 cm^{-1} (C=C) was assigned to the trigonal mode of the phenyl ring. The bands at 1359, 1273 and 1173 cm^{-1} (CH_2) were assigned to the tropine ring and/or protein and aliphatic amino acids present in the OF and the scattering at 1000 cm^{-1} is characteristic of cocaine's asymmetric stretch of the phenyl ring.

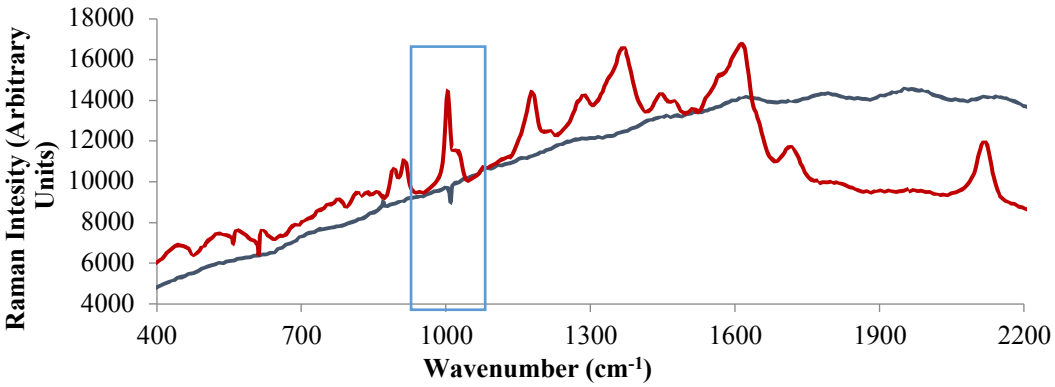


Figure 2.8. Raman spectra (Handheld Raman 532 nm, Rigaku) of control SOF (▬) and cocaine in SOF (▬) at 1 mg/mL using Substrate 2.

2.3.2.2.1 SOF sample to Substrate 2 ratio

The sample to substrate ratio was evaluated because the enhancement of Raman scattering is determined by the interaction between the analyte and the hot-spots in the NPs. Figure 2.9 illustrates the difference in scattering at a wavenumber of 1000 cm^{-1} for cocaine in SOF at 1 mg/mL, when samples were mixed in different proportions with the substrate (ratios analysed were 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10).

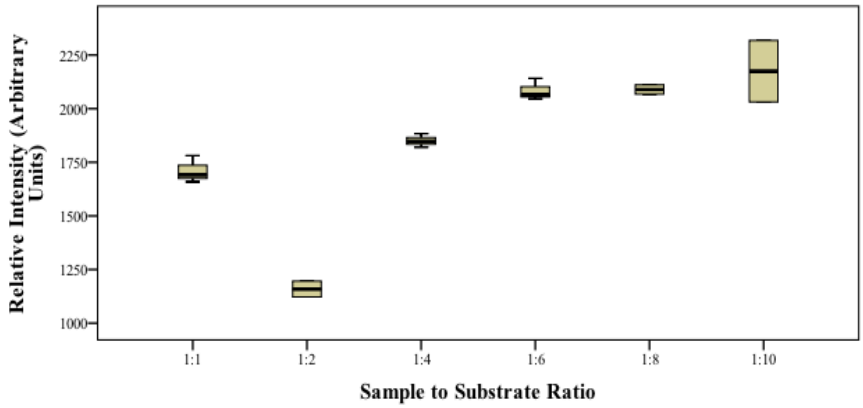


Figure 2.9 Box and whisker plot of the effect of SOF sample to substrate ratio on the intensity of Raman signal for the analysis of cocaine in SOF. Sample to Substrate 2 ratios of 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10. Samples at concentration of 1 mg/mL cocaine in SOF. Time of interaction of 30 min. The box represents the interquartile range with the median. The whiskers extend to the 10^{th} and 90^{th} percentiles.

A Kruskal-Wallis H test showed that there was a significant statistical difference in Raman scattering between the different sample to substrate ratios, $H(5) = 12.42$, $p = 0.03$. Although, when performing post hoc tests (Mann-Whitney and Bonferroni) to determine which sample to substrate ratio was different, no significant statistical difference was observed within the samples ($p = 0.2 – 1$). A Jonckheere's test revealed a significant trend in the data: the median Raman intensity increased with an increase in the amount of substrate, $J = 80$, $z = 3.37$, $r = 0.87$.

In addition to statistical analysis, it was observed that at bigger ratios (1:10), the peaks were more resolved that at lower ratios (1:1). A ratio of one to ten was kept during the development of this study. This observation could be related to the increase in the number of NPs and therefore, the probability of the cocaine to get adsorbed on the NPs. It could also be related to a decrease in noise from the surrounding cocaine particles being adsorbed on neighbours NPs, this because at bigger ratios the adsorbed cocaine molecules are more distant from one another.

Higher concentrations of NPs were not evaluated (sample to substrate ratios >10) as an over-agglomeration of the NPs could occur. Consequently, reducing the number of hot-spots and available surfaces for the analyte to be adsorbed. However further studies need to be conducted to confirm the decrease of SERS activity when using higher sample to substrate ratios

2.3.2.2.2 Time of interaction between SOF samples and Substrate 2

The interaction between samples of SOF (1 mg/mL) containing cocaine and S2 was evaluated at three different time periods (5 min, 30 min and 1 hour) to determine whether

SERS enhancement was time dependent. An increase in the time of interaction between the sample and the substrate could lead to an increase in the probability of deposition of cocaine molecules on the substrate's surface, i.e. more cocaine molecules could be enhanced by the NPs' plasmoid.

Figure 2.10 illustrates the difference in Raman scattering (peak at 1000 cm^{-1}) from a sample of 1 mg/mL cocaine in SOF when Raman spectra were collected at 5, 30 and 60 minutes after interaction with the substrate. The results showed an increase in scattering when samples of SOF containing cocaine were left in contact with the substrate for a longer period of time.

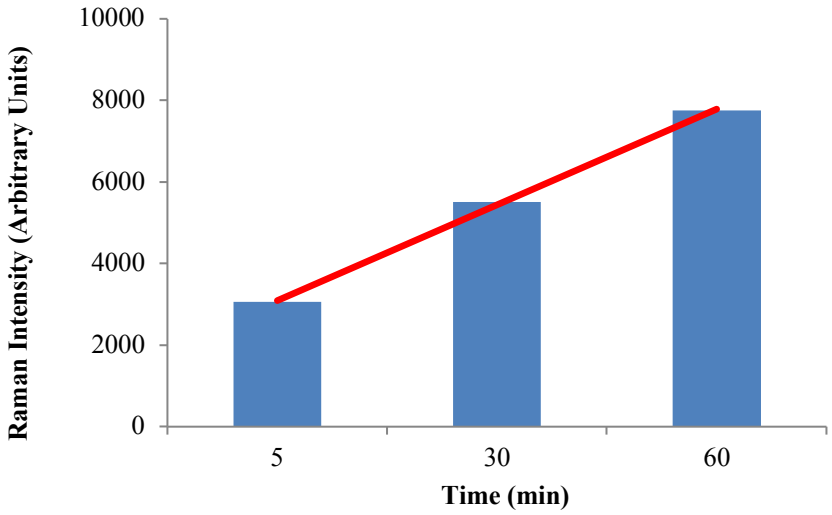


Figure 2.10 Effect of time of interaction between Substrate 2 and SOF samples containing cocaine (1 mg/mL) on the intensity of Raman signal for the analysis of cocaine in SOF. Raman spectra collected using the Rigaku handheld Raman 532 nm.

The results obtained showed that SERS enhancement was proportional to the time of interaction between the SERS substrate and the sample, thus indicating that there was a higher probability that cocaine molecules interacted with the electromagnetic field of the hot-spots over time. The intensity of the Raman signal of samples analysed after 5 and 30 minutes were 39% and 71% of the Raman signal of the samples analysed after one hour

of interaction. Since the difference in the intensity of Raman signal between the interaction at 30 minutes and one hour was less than 50%, it was decided that 30 minutes of interaction was an appropriate time to allow for the adsorption of cocaine on the surface of the NPs (S2). All SOF and tongue tissue samples were subsequently evaluated after 30 minutes of the mixture with the substrate.

2.3.2.2.3 Limit of Detection

The SERS spectra collected for the analysis of linearity and LOD were collected using an increased exposure time (14 seconds rather than 4) in order to be able to obtain more sensitive results. The time of exposure is related to the time that the laser hits the sample, which increases the amount of Raman spectra that are recorded and accumulated to one spectrum. Hence, the intensity of the Raman signal (sensitivity) was proportional to the amount of spectra accumulated.

LOD in this study was 0.1 mg/mL cocaine in SOF. Comparison between the LOD presented in this section and previously reported values (Farquharson et al. 2011; Dana et al. 2015) indicated that a sample pre-treatment should be conducted in order to obtain lower LOD of cocaine in SOF. Farquharson et al. (2011) and Dana et al (2015) reported LOD ranging 25-50 ng/mL for the detection of cocaine, which were achieved after solid phase extraction (SPE). Dana et al. (2015) reported that un-extracted samples of cocaine in OF at 10 μg/mL or below only produced a background spectrum.

As mentioned above, SOF influenced the sensitivity of the results; therefore, it can be assumed that lower limits of detection could have been obtained if a sample pre-treatment (like SPE) had been conducted. However, sample pre-treatment was not considered in

this study as the aim of the research was to evaluate the direct analysis of cocaine in SOF with no sample pre-treatment.

2.3.2.2.4 Linearity

Three regression lines with mean concentrations of cocaine in SOF were obtained for a concentration range of 0.1-1.0 mg/mL in SOF using six concentration points: (1) $y = 77.12x - 19527$; $R^2 = 0.954$, (2) $y = 84.270x + 18372$; $R^2 = 0.837$ and (3) $y = 66.002x + 32253$; $R^2 = 0.797$. Mean values were calculated based on a minimum of three replicates at each concentration point. Calibration lines and residual plots are shown in Figure 2.11. Random scattering of the residuals were observed with all values falling within the corresponding values of $\pm t_{(0.95\ np-2)}$, which indicated a linear correlation. The F-test (α = 0.5%) indicated that the data was homoscedastic and therefore the variance around the regression line was uniform ($p < 0.05$). The F-test and the residual plot confirmed the linear regression of the model.

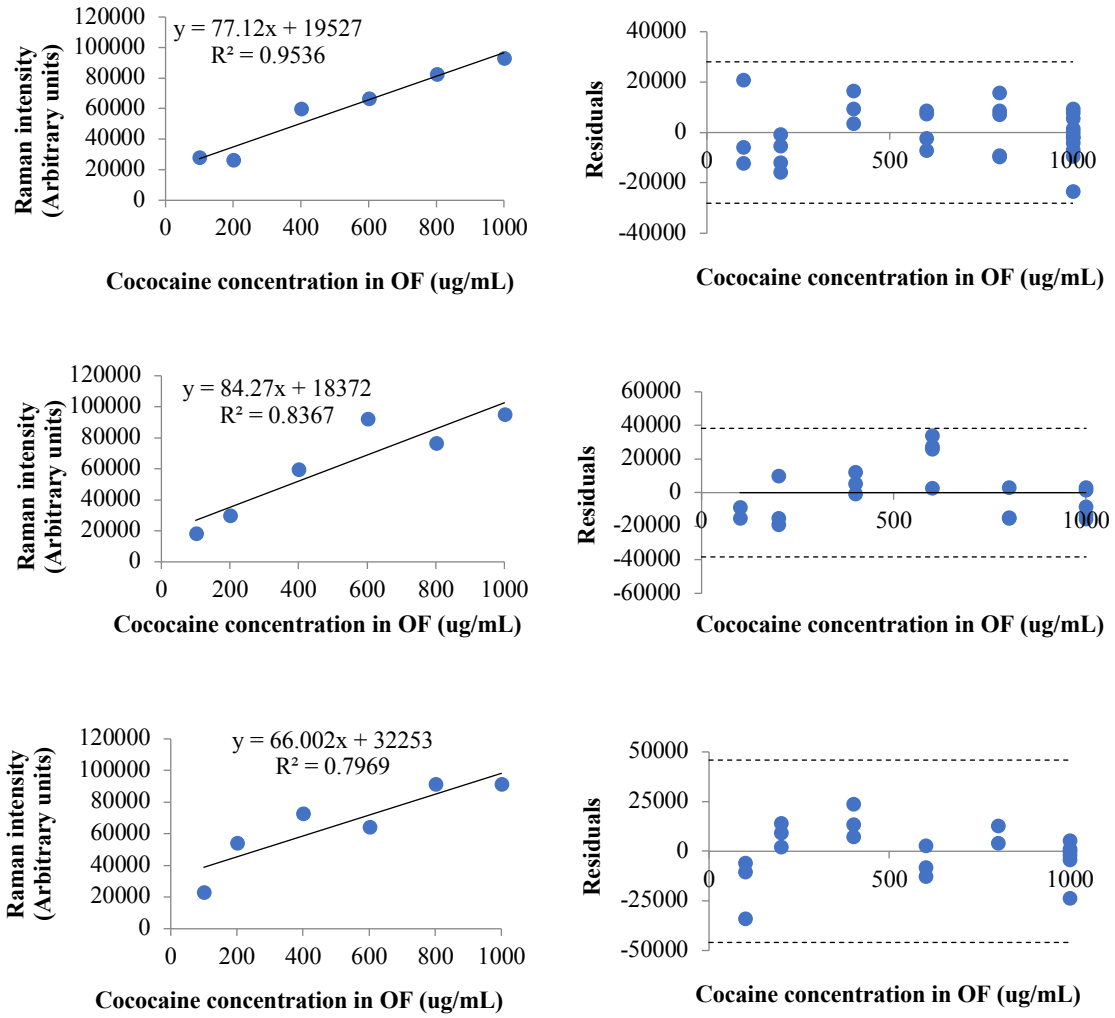


Figure 2.11 SERS calibration lines and residual plots for cocaine in OF using Subsrate 2. Data represents mean values (n>3). Doted lines (-) represents $\pm t_{(0.95,\ np-2)}$.

2.3.2.2.5 Accuracy and precision

Intra-day precision data are summarised in Table 2.3. The intra-day precision values were > 25% for the low (0.1 mg/mL) concentration, which was above the acceptable value of ± 20% (USP 2011). Precision values were < 11% for the medium (0.4 mg/mL) and high (1 mg/mL) concentrations. Intra-day accuracy was also above the acceptable value of ± 20% (USP 2011) and varied between concentrations and analysis. In general, inter-day accuracy was higher than 40% and ranged from 47-140%.

Table 2.3 Intra-day and inter-day data for analysis of cocaine in SOF using Substrate 2. Analysis conducted using a Rigaku 532 nm handheld Raman spectrometer.

		Concentration (μg/mL)		
		1000 (H)	400 (M)	100 (L)
Intra-day 1-1 (*n* = 6)	Mean	91457	73027	23077
	SD	10153	8308	15055
	SE	4145	4796	8692
	%RSD	11	11	65
	Accuracy (%)	89	140	86
Intra-day 1-2 (*n* = 6)	Mean	94918	59609	18148
	SD	8549	6513	4468
	SE	3823	3760	3159
	%RSD	9	11	25
	Accuracy (%)	97	102	47
Intra-day 2 (*n* = 6)	Mean	40696	20929	2455
	SD	832	1324	115
	SE	588	936	81
	%RSD	2	6	5
	Accuracy (%)	97	133	94
Inter-day (*n* = 3)	Mean	75690	51188	14560
	SD	30355	27050	10769
	SE	17526	15618	6218
	%RSD	40	53	74

SD: Standard deviation, SE: Standard error, %RSD: Percentage relative standard deviation. H: High concentration, M: Medium concentration and L: Low concentration.

The inter-day precision for cocaine in OF was higher than 40% and above the acceptable value (± 20%) Inter-day precision values were 40, 53 and 74% for the high, medium and low concentrations respectively.

2.3.2.2.6 Matrix effect in SERS analysis

Matrix effect is the effect on an analytical method caused by all other components of the sample except the specific compound to be quantified (Semeraglia 2002). This effect was evaluated to determine the interference of SOF in the analysis of cocaine by SERS. Most SERS suppliers suggest the pre-treatment of OF samples by extracting the analyte of interest and eliminating any other interferent substances in order to lower the LOD of the method. Since there is no clear evidence of interference of the SOF in the analysis of drugs by SERS, an evaluation of the dilution factor and therefore the matrix effect of the sample was conducted. Few discussions were reported by Dana et al. (2015) describing the differences in limits of detection for the detection of cocaine before and after sample pre-treatment, i.e. elimination of the interference of OF from the sample. However, no discussion on the effect of the OF in the Raman scattering of compounds (e.g. cocaine) was given.

When SOF samples were diluted with water and samples were prepared to a final concentration of 1 mg/mL of cocaine, the Raman intensity of the cocaine peak (Wavenumber at 1000 cm^{-1}) increased linearly to a maximum of 59 % of the initial value (dilution factor of 1:34) (see Figure 2.12). These results confirmed that the matrix affects the final response of the analyte, i.e. the presence of SOF in the sample interferes with the scattering of cocaine. The results also suggested that samples of SOF containing cocaine or any drug should be diluted in order to increase the sensitivity of the SERS analysis.

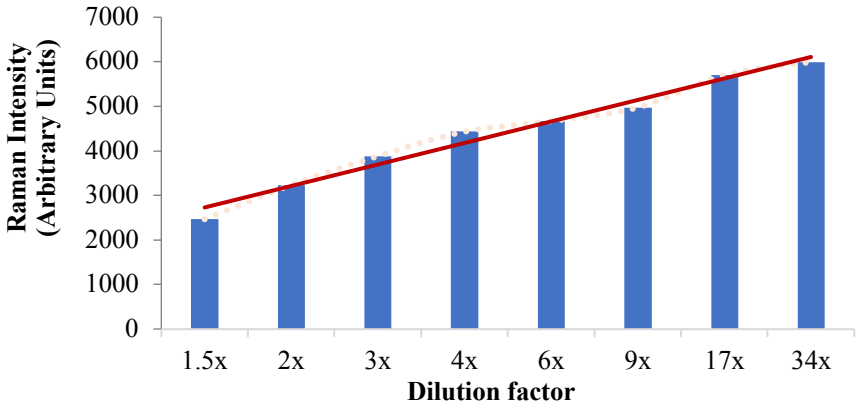


Figure 2.12 The effect of SOF on the intensity of the Raman signal for the analysis of cocaine using Substrates 2. Dilution factors of 1.5, 2, 3, 4, 6, 9, 17 and 34 were evaluated using water as diluent. Samples were at a concentration of 1 mg/mL of cocaine. The Substrate 2 to sample ratio was 10:1 in OF. Time of interaction was 30 min. Correlation coefficient $R^2 = 0.98$ ($y = 483x + 2248$).

The interference of SOF in the SERS analysis could be attributed to a competition between the SOF constituent molecules and the cocaine molecules to be adsorbed onto the surface of the NPs. As the number of molecules from the components of the SOF increases, the molecules of drug (cocaine) have less chance to be adsorbed onto the NPs, thus decreasing the scattering of the cocaine molecules.

While the matrix effect of the SOF was confirmed and the dilution of the samples suggest an increase the sensitivity of the SERS analysis, it is important to note that larger dilutions are a disadvantage because limits of detection will need to be increased to detect such diluted samples.

2.3.2.2.7 Analysis of tissue containing cocaine

Despite of the Raman scattering enhancement obtained for the analysis of cocaine in SOF samples sing S2, no Raman scattering of cocaine from tongue tissue samples was obtained using this substrate. Conditions such as: (1) sample to substrate ratios, (2) instrument conditions, (3) physical state of the substrate (liquid and dry substrate) and (4)

physical state of the sample (fresh tissue and dry tissue), were evaluated to decrease the fluorescence from molecules in the tissue and obtain Raman scattering unsuccessfully. No scattering was obtained for cocaine and/or any of the components of the tissue such as proteins and lipids.

2.3.2.3 *Analysis of synthetic oral fluid and tissue samples using Substrate 3*

Initially, a solution of control SOF and S3 in a ratio 1:1 was evaluated to determine any interferent peaks. Following the control spectra, the SERS spectra of cocaine in SOF was obtained at concentration of 1 mg/mL to evaluate the enhancement of the Raman signal when using S3 (Figure 2.13). A time of interaction of 30 minutes was used based on the results from S2. This time was allowed to provide enough time for the molecules of cocaine to be adsorbed onto the substrate's surface.

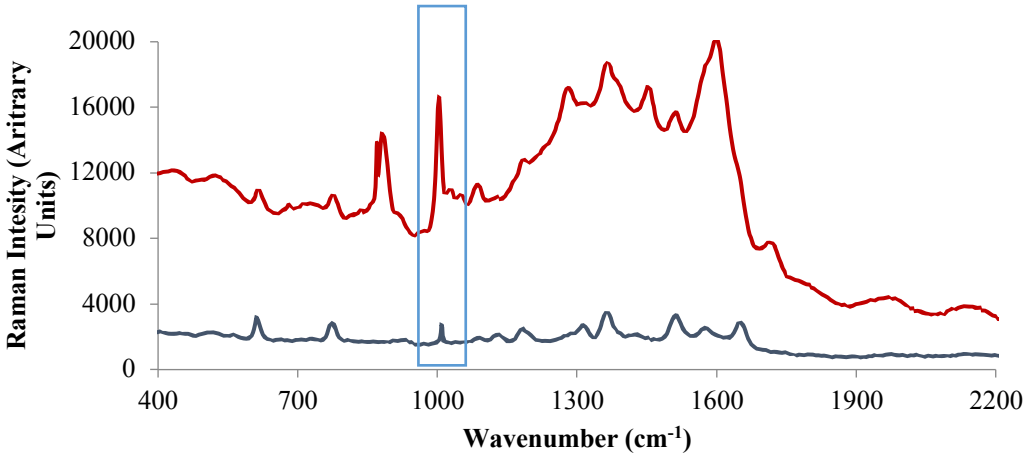


Figure 2.13 SERS spectra (Handheld Raman 532 nm, Rigaku) for control SOF ( )and 1 mg/mL cocaine in SOF ( ) using Substrate 3. Sample to substrate ratio was 1:1.

The spectra of control SOF and SOF containing cocaine (Figure 2.13) showed the same predominant bands as those obtained with S2 (Figure 2.8): 1704 cm^{-1} (C=O stretching) from the tropine ring stretch, the symmetric and asymmetric phenyl ring, the trigonal phenyl ring breathing mode, and the ester carbonyl stretch. The band at 1605 cm^{-1} (C=C

amide I) was assigned to proteins present in the SOF. The band at 1605 cm^{-1} (C=C) assigned to the trigonal mode of the phenyl ring. The bands at 1359, 1273 and 1173 cm^{-1} (CH_2) were assigned to the tropine ring and/or protein and aliphatic amino acids present in the SOF and the scattering at 1001 cm^{-1} characteristic of cocaine asymmetric stretch of the phenyl ring.

2.3.2.3.1 SOF Sample to Substrate 3 ratio

Figure 2.14 illustrates the change in scattering at wavenumber of 1001 cm^{-1} for cocaine in SOF at 1 mg/mL when samples were mixed in different proportions with the substrate S3 (ratios analysed were 1:1, 2:1, 4:1, 6:1, 8:1 and 10:1).

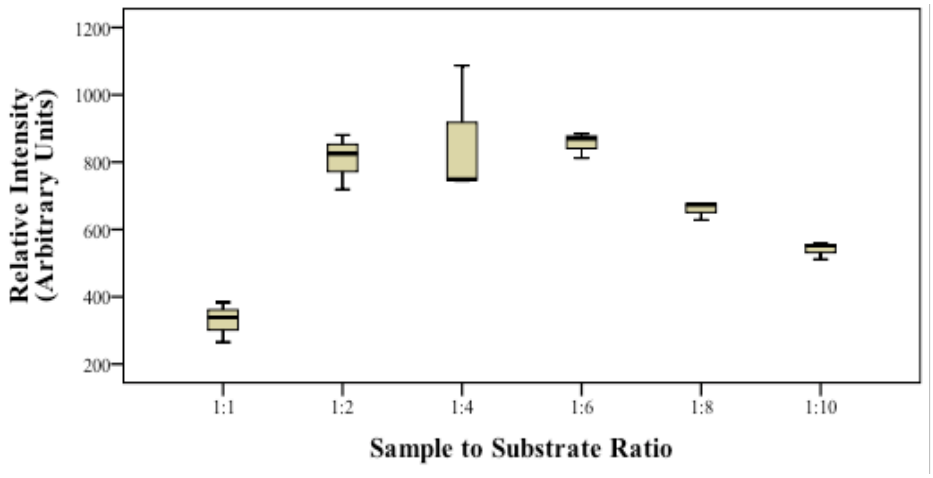


Figure 2.14 Box and whisker plot of the effect of SOF sample to substrate ratio on the intensity of Raman signal for the analysis of cocaine in SOF. Sample to Substrate 3 ratios of 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10. Samples at concentration of 1 mg/mL cocaine in SOF. Time of interaction of 30 min. The box represents the interquartile range with the median. The whiskers extend to the 10^{th} and 90^{th} percentiles.

A Kruskal-Wallis H test showed that there were statistical significant differences in Raman scattering between the sample to substrate ratios, $H(5) = 14.85$, $p = 0.01$. It appeared that intensity of the Raman signal was significantly different (following port hoc tests Mann-Whitney and Bonferroni) for ratios 1:1 and 1:6 ($p = 0.04$) only. Because most (93%) asymptotic significance values were > 0.05 it was concluded that there were

no differences between most sample to substrate ratios. A Jonckheere's test revealed that the intensity of Raman signals was the same across all ratios, J = 62, z = -0.42, r = -0.11. Additional to statistical analysis, no differences were seen following visual analysis on peak shape and signal to noise. Hence, experiments were undertaken using sample to substrate ratio of 1:1.

2.3.2.3.2 Limit of detection

The LOD of cocaine in SOF using S3 had a value of 1 mg/mL and was obtained with an increased exposure time (14 seconds) in order to obtain more sensitive results. The LOD obtained for cocaine in SOF using S3 was considerable higher ($5x10^5$ times) than the LOD reported by Wang et al. (2015) for the analysis of Rhodamine B in water (2 ng/mL). These remarkable differences in sensitivity of S3 could have been the result of unsuccessful synthesis of this substrate (S3) and the effect that SOF had on SERS analysis as was mentioned above.

A comparison between the LOD obtained for the detection of cocaine in SOF using S2 (0.1 mg/mL) and S3 (1 mg/mL) showed that the SERS activity of S3 was considerably lower than the S2. The SERS scattering of cocaine using S2 was four times the scattering of that for cocaine using S3 at the same concentration of cocaine in SOF (1 mg/mL). This result could be attributed to a decrease in hot-spots of the S2. The fact that the particle size of S3 (22 ± 4 nm) was lower than for S2 (50 ± 26 nm), indicated that the core-shell could have affected the electromagnetic cloud (hot-spot formation) and therefore the SERS activity of the S3.

2.3.2.3.3 Linearity, accuracy and precision

Linearity, accuracy and precision were not evaluated with this substrate because the LOD was ≥ 1 mg/mL cocaine in SOF

2.3.2.3.4 Analysis of tissue containing cocaine

No studies were conducted on the tongue tissue using S3 because SERS enhancement was not observed at concentrations below 1 mg/mL cocaine in SOF. It was concluded that if cocaine at 1 mg/mL in SOF produced low enhancement when analysed with S3, no enhancement would be obtained from tissue containing cocaine (cocaine concentrations in homogenate tissue ranged between 25–870 ng/mL) (Reichardt 2014).

2.3.2.4 *Analysis of synthetic oral fluid and tissue samples using Substrate 4*

The mixture of S4 (magnetic NPs) with the highest concentration of cocaine solution (10 mg/mL cocaine in ethanol), resulted in no visible differences in colour or magnetic properties of S4. The agglomeration of magnetic NPs was achieved by the use of a magnet. This observation indicated that agglomeration of NPs and therefore formation of hot-spots were not affected by the presence of cocaine and/or ethanol. Figure 2.15 shows the representative SERS spectra of S4 with ethanol and cocaine in ethanol at a concentration of 10 mg/mL. No Raman scattering was obtained for S4 mixed with ethanol, therefore any scattering at Wavenumber of 1000 cm^{-1} was further attributed to the presence of cocaine.

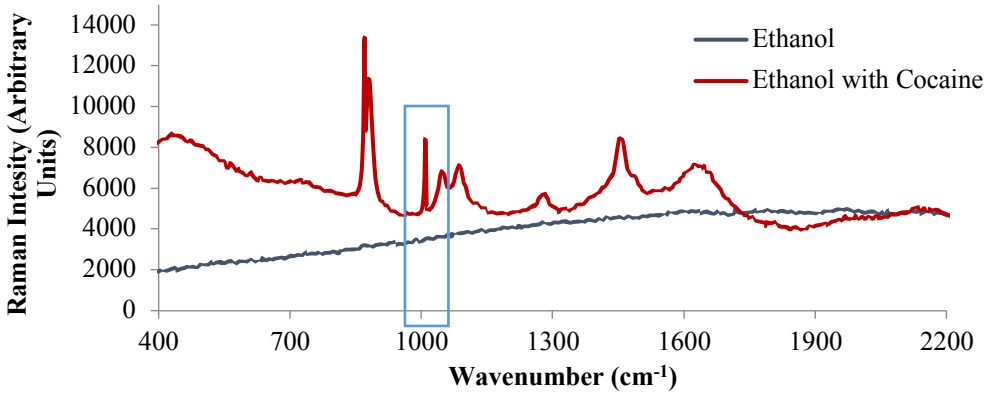


Figure 2.15 SERS spectra (Handheld Raman 532 nm, Rigaku) for ethanol ( ) and 10 mg/mL cocaine in ethanol ( ) using Substrate 4. Sample to substrate ratio of 1:1.

SERS Spectra of cocaine in methanol (Figure 2.15) showed the characteristic scattering of cocaine tropine ring stretch, symmetric and asymmetric phenyl ring breathing modes, C-phenyl stretch, trigonal phenyl ring breathing mode and ester carbonyl stretch (870, 1008, 1042, 1265, 1448 and 1600 cm^{-1}). There were evident changes in frequency compared with the reported bands for cocaine (872, 999, 1026, 1273, 1597 and 17116 cm^{-1}) (Farquharson et al. 2011). These changes could be attributed to the extent to which each vibrational mode interacted with the surface and plasmon (hot-spots) of the magnetic substrate.

Detection of cocaine in SOF samples containing cocaine could not be obtained from the highest standard of cocaine in SOF (1mg/mL); therefore, it was determined that the LOD for the analysis of cocaine using S4 was 10 mg/mL. This LOD was 10^7 times higher than that reported by Yang et al. (2014, 2015), where the LOD for benzoylecgonine in OF was reported at 29 ng/mL. These enormous differences could be attributed to the quality of the synthesised particles and therefore to the synthesis procedure. During the preparation of the Substrate 4, it was noted that many details on the conditions under which each step of the synthesis was performed were omitted in the publication. Even though the physical characteristics of the magnetic substrate matched those described by

Wang et al. (2014; 2015), e.g. the reddish colour and magnetic effect, the activity of the obtained SERS substrate was not achieved.

Evaluation of linearity, sample to substrate ratio and matrix effect was not conducted for the analysis of cocaine in SOF using S4 because of the high LOD obtained.

2.3.2.4.1 Analysis of tissue containing cocaine

No studies were performed on the tongue tissue because SERS enhancement was not observed at concentrations below 10 mg/mL of cocaine. It was concluded that if a low enhancement was produced when analysing a concentration of cocaine at 10 mg/mL, no enhancement would be obtained from the tissue containing cocaine (cocaine concentrations in tissue range 25-870 ng/mL) (Reichardt 2014).

The application of SERS in drug detection has a number of limitations, including the dependency on the morphology, optical and electromagnetic properties of the substrate in use. Enhancement of the resonance of the NPs depends upon the adsorption of the analyte to the surface of the substrate. Reproducing the analysis is problematic because of the difficulty of having an equal number of NPs and consequently hot-spots formed each time, and differences in these factors may lead to changes in the intensity of the scattering at specific concentrations of analyte. Finally, and not related to the SERS substrate, the sensitivity of the Raman instrumentation and the limit of detection can add further limitations.

2.4 CONCLUSIONS

The synthesis of four different SERS substrates (S1 to S4) was achieved through various routes: chemical deposition, reduction and seed growth. These four substrates were subsequently used in the SERS analysis of cocaine in SOF and porcine tongue tissue. The synthesised substrates were silver and gold-based substrates, which are the most common metallic NPs used as SERS substrates. Selection of the synthesis procedures was based on reported LOD values for these substrates, as well as the feasibility of the synthetic procedure.

Characterisation of all four synthesised substrates indicated that synthesis of silver and/or gold NPs were successful. Low differences were seen (< 29%) when the particle sizes were compared with the reported values for each synthesised substrate. The mean particle size for the S1 (110 ± 20) were within the range of the reported value (155 ± 75)(Wrona et al. 2015). Average particle size for S2 was 50 ± 26 nm compared with the reported 35 ± 5 nm (Feng 2015). However, the major percentage of S2 ranged 30-50 nm. Particle size for S3 was 22 ± 4 compared with the reported 25 nm (Wang et al. 2015) and 13 ± 3 nm compared with the reported 11.7 ± 2.7 nm for S4 (Yang et al. 2015). In addition to the particle size, physical characteristics such as appearance, i.e. morphology of the particles, colour of the final substrate and magnetic properties (S4 only) indicated that all substrates were successfully synthesised. From the above, it was concluded that all substrates could form hot-spots and therefore enhance the Raman signal of drugs in OF and tongue tissue.

SERS spectra of cocaine in SOF were successfully collected at the LOD of 0.1 mg/mL and 1 mg/mL using S2 and S3 respectively. A LOD of 10 mg/mL cocaine in ethanol was

obtained with S4. No spectra were obtained for solutions of cocaine in SOF or ethanol when using the S1. Data from this chapter showed that silver NPs obtained by hydroxylamine reduction (S2) had the highest SERS activity when compared with the other substrates.

The quantitative SERS analysis concluded that cocaine could only be detected at levels above 100 μg/mL in SOF using S2 using a handheld Raman spectrometer with 532 nm laser exposure. Even though the LOD was highly increased by the use of this substrate (10^4), this method (LOD) would not allow the detection of cocaine in real OF samples as concentration of cocaine in OF range 0-8.6 μg/mL (Kato et al. 1993, Cone 2012, Reichardt 2014). Likewise, it would not allow the *in vitro* monitoring of the release of cocaine from oral drug depots (tissue) into SOF, where maximum concentration of cocaine in tissue or SOF are expected to range 5-8 μg/mL. Although linear regression was obtained for the method using S2 (Mean $R^2 = 0.863$), the results obtained demonstrated that accuracy (> 25 %) and precision (< 40 %) were below the values recommended by the Unite States Pharmacopeia for the validation of Raman spectroscopic methods (USP 2011). Future analysis using more sensitive Raman instrumentation is recommended to increase the sensitivity, precision and accuracy of this method.

Analysis of tongue tissue using S1, S3 and S4, was not conducted because of the high values of LOD obtained from the SOF sample analysis with each of these substrates. Analysis of tissue using S2 did not show any visible bands from either cocaine or tissue constituents (proteins, lipids, etc.). Therefore, it can be concluded that cocaine cannot be detected using any of the SERS substrates described in this chapter and the Rigaku First Guard handheld Raman spectrometer.

Chapter 3

EVALUATION OF COMMERCIAL SERS SUBSTRATES FOR THE DETECTION OF COCAINE IN SYNTHETIC ORAL FLUID AND PORCINE ORAL TISSUE USING PORTABLE RAMAN SPECTROSCOPY AND RAMAN MICROSCOPY

3.1 INTRODUCTION

In latest years, various research groups and manufacturers have made a great effort in improving Raman instrumentation and developing Surface Enhanced Raman Spectrometry (SERS) substrates for applications in drug testing (Farquharson et al. 2011, Dana et al. 2015, Yang et al. 2015). The vast majority of these SERS substrates has been developed by originally synthesising silver and/or gold nanoparticles (NPs) in the form of colloidal metal suspensions, i.e. metal NPs in suspension such as those described in Section 2.2.3 (e.g. synthesis of silver NPS via hydroxylamine reduction) (Feng 2015, Wang et al. 2015, Yang et al. 2015).

The results presented in Chapter 2 indicated that SERS substrates obtained via hydroxylamine reduction could be used for the detection and quantification of cocaine in synthetic oral fluid (SOF) at limits of detection (LOD) of 100 μg/mL. However, this LOD did not provide the required sensitivity for the analysis of cocaine in SOF or tissue at physiological levels (0-9μg/mL) without the use of sample pre-treatment.

In order to produce hot-spots and increase the SERS activity of a substrate, an effective aggregation of the NPs need to be achieved, i.e. NPs should be near to one another. Recent publications have proved that SERS substrates can be fabricated with more controlled aggregation of the NPs by using more elaborate procedures such as the immobilisation of metallic NPs on solid surfaces (paper or silica). Some of the common techniques used to immobilise NPs include the deposition or inject printing, the *in situ* growth (synthesis of NPs directly on solid surfaces) or the lithographic techniques (Sharma et al. 2012, Mosier-Boss 2017). These procedures have the advantage of producing commercial SERS

substrates of high quality at an affordable cost, which could benefit the implementation of the Raman technique in drug testing programs (Thomson 2002, Radzol et al. 2012).

Additional to the activity of the SERS substrates, the characteristics of the Raman instrumentation plays an important role in the sensitivity of the method (Kiselev et al. 2016). The selection of the correct excitation laser, the power of the laser and improvement of the optics allow more sensitive Raman measurements (Section 1.11). Raman spectrometers can be coupled to a microscope to enable depth analysis of a sample as the incident excitation laser can be focus on the sample and the scattered light can be collected more accurately. Laboratory based Raman microscopes offer significant advantages over portable and handheld Raman spectroscopes because of the improved optics and detectors, which result on an increased sensitivity of the analysis (Turrell and Corset 1996). Raman microscopy has been used for the analysis of molecules (proteins, pharmacologically relevant molecules and vitamins) in biological matrices for medical diagnosis (Kiselev et al. 2016). Some examples on the application of Raman microscopy in the analysis of drugs in biological tissues are: (1) the label-free imaging of erlotinib distribution and metabolism in colon cancer cells (El-Mashtoly et al. 2014), and (2) the use of Raman microscopy in imaging of omega-3 fatty acids and saturated lipids in living cells (Freudiger et al. 2008).

This chapter describes the use of two commercial SERS substrates for the detection of cocaine in SOF and their comparison to the use of a homemade substrate (silver NPs obtained by the hydroxylamine reduction, Chapter 2) using a portable Raman spectrometer. This analysis was developed to decrease the LOD of cocaine in SOF obtained with homemade substrates (described in Chapter 2) and handheld Raman spectroscopy. As was previously mentioned, commercial substrates are commonly

prepared by complex and accurate techniques that could have NPs with an increased number of hot-spots and therefore which could provide more sensitive methods. An evaluation of the sensitivity of the analysis and the quantification of cocaine in SOF was also conducted.

Additional to the analysis of cocaine using portable Raman spectrometry, Raman microscopy was used to increase the sensitivity of the Raman detection and to evaluate further the presence of cocaine in porcine tongue tissue. Even though, portable Raman instruments can easily be used by unskilled individuals and offer the advantage of allowing the analysis of samples on site without the need to transport the samples to the laboratory, portable instruments are not as accurate and sensitive as laboratory-based instruments.

3.1.1 AIM AND OBJECTIVES

3.1.1.1 *Aim:*

The aim of this chapter was to evaluate the use of commercial SERS substrates using portable Raman spectroscopy and the use of Raman microscopy for the detection of cocaine in porcine tongue tissue.

3.1.1.2 *Objectives:*

- Assess the use of commercial SERS substrates in the detection and quantification of cocaine in SOF using portable Raman spectrometer

- Compare the response of Raman signal obtained with commercial SERS substrates with the response obtained using a home-made SERS substrate in the detection of cocaine in SOF.
- Evaluate the use of Raman microscopy for the detection of cocaine from porcine tongue tissue.

3.2 METHODS

3.2.1 Materials

The reagents used to synthesise the silver substrate were purchased as described in Section 2.2.

Crack cocaine was provided by John Ramsey, TICTAC Communications, St Georges Medical School, University of London. Cocaine hydrochloride was purchased from Sigma-Aldrich (Pool, UK). Internal standard cocaine-d_3 was purchased from LTG Standards. Bis-(trimethylsilyl)-trifluoroacetamine (BSTFA) + 1% trimethylchlorosilane (TMCS) and β-glucuronidase, Type H-1 (from *Helix pomatia*) were purchased from Sigma-Aldrich. Ammonia solution (33%), hydrochloric acid (37%) and phosphate buffer (1.0M, pH 6.0) were purchased from Fisher Scientific (Loughborough, UK). TELOS® SPE cartridges (3 mL volume) with 130 mg H-CX sorbent were purchased from Kinesis (Cambridgeshire, UK).

Porcine tongues were purchased from LFB Meats, Bournemouth, UK.

Test sticks gold substrates (Thermo Scientific) were purchased from Hamamatsu. Tac Pack^{TM}-P substrates (Paper strips) were donated by BWTek.

TELOS® SPE cartridges (3 ML volume) with 130 mg H-CX sorbent were purchased from Kinesis (Cambridgeshire, UK).

3.2.2 Instrumentation

Portable Raman spectrometer iRaman Plus (B&W Tek, UK) with a fibre optic probe coupled to a video microscope and a charge coupled device (CCD) detector. The spectrometer was equipped with 785 nm laser power, >320 mW (420 mW max) laser output power and laser control of 0 to 100%. The camera had an effective pixel size of 14 μm x -0.9 mm and magnification objectives of 10x, 20x, 40x and 100x; this was used to focus the laser beam on the sample. Raman spectra were collected over the wavenumber range of 200-3200 cm^{-1} and spectral resolution of 2.4 cm^{-1}. Each spectrum was the sum of a minimum of three scans. Data collection was conducted by the researcher at Bournemouth University.

Raman Microscopy measurements were performed using a DXR_{TM} dispersive Raman microscope (Thermo Scientific, UK) equipped with 534 nm and 780 nm laser power and a charge coupled device (CCD) detector. The slit aperture and spot size used were 50 μm and 2.1 um respectively. Raman spectra were collected over the wavenumber range of 200-2000 cm^{-1} and spectral resolution of 5 cm^{-1}. Data collection was conducted in collaboration with Thermo Fisher Scientific at Thermo Fisher Scientific, Hemel Hempstead – UK.

Quantification by gas chromatography couplet to mass spectrometry (GC-MS) was conducted using a Varian 2200 Gas Chromatographer coupled with a Varian Saturn 200 ion-trap detector (Agilent Technologies, Stockport, UK). Chromatographic separation was conducted using a BPX5 fused silica capillary column (30 m x 0.25 mm i.d. 0.25

μm) with 5% phenyl – 95% methyl-polysiloxane stationary phase (SGE Analytical Science, Ringwood, Australia). Helium was used as carrier gas at flow rate 1.0 mL/min. Data collection was conducted by the researcher at Bournemouth University.

3.2.3 Synthetic oral fluid sample preparation

SOF was prepared using the Cozart Biosciences protocol (2008) "Production of Synthetic Saliva" (Appendix A).

Samples of SOF with cocaine and without cocaine (control SOF) were used. Samples of SOF containing cocaine were prepared by serial dilutions of a stock solution (20 mg/mL cocaine hydrochloride in ethanol). The final concentration of the test samples was 0.1, 0.5, 1, 5, 10 and 20 mg/mL of cocaine hydrochloride in SOF.

3.2.4 Tissue sample preparation

Details on the exposure of tongue tissue to crack cocaine and tissue sample preparation were outlined in section 2.2.8 Sections of porcine tongue tissue with thickness at 0.02 mm, 2 mm and 5 mm containing cocaine were analysed using the Thermo Scientific DXR Raman microscope.

3.2.5 Analysis of synthetic oral fluid samples - Acquisition of Raman spectra using portable Raman spectroscopy

Test samples of control SOF and SOF containing cocaine (range 0.1-20 mg/mL) were analysed using the iRaman Plus portable Raman instrument with and without the use of SERS substrates.

In order to develop the SERS analysis, three different substrates (S2, S5 and S6)[1] were assessed for their use in the detection of cocaine in SOF. The substrates used in this chapter were either silver or gold NPs (Table 3.1). The synthesis and characterisation of S2 were described in sections 2.2.3 and 2.2.4 respectively. Substrates S5 and S6 are commercially available and were donated by B&WTek and Thermo Fisher Scientific respectively.

Table 3.1 Details of gold and silver substrates used for the detection of cocaine using the portable iRaman Plus 785 nm spectrometer.

Substrate	Substrate name	Substrate base	Physical state	Description	Supplier
S2	Hydroxylamine	Silver	Liquid	NPs in suspension	Homemade
S5	Paper	Gold	Solid	NPs deposited on paper	B&W Tek
S6	Silicon	Gold	Solid	NPs deposited on silicon pieces	Thermo Scientific

Homemade: substrate synthesised in the laboratory at BU, NPs: Nanoparticles, S2: Substrate 2, S5: Substrate 5 and S6: Substrate 6.

Raman spectra were collected by directly exposing the samples to the laser beam. SERS spectra were obtained by measuring the scattering of the samples with the respective substrate: (1) S2 was used by mixing the test samples with the substrate in a

[1] The use of substrates S1 and S3 were described in Chapter 2.

1:10 ratio and analysed following a time of interaction of 30 min. (2) S5 was used by immersing the substrate into the corresponding sample. The sample measurements were conducted after five minutes, when the paper was dry. (3) S6 was used by directly applying a drop of the test sample on the surface of the substrate and sample measurements were collected after five minutes, when the sample was dried. Raman spectra were collected at various exposure times of the substrates to the laser beam (1-20 s), laser power percentage (20-100%) and number of scans (4-8) (Table 3.2).

Table 3.2 Conditions used to analyse cocaine in SOF using SERS and iRaman Plus 785 nm spectrometer.

Substrate	Substrate name	Substrate base	Laser exposure (%)	Laser exposure (mW)	Exposure time (s)	Number of Scans
S2	Hydroxylamine	Silver	100	320	20	8
S5	Paper	Gold	20	64	1	4
S6	Silicon	Gold	100	320	10	8

Laser Exposure: Approximate values, s: seconds, S2: Substrate 2, S5: Substrate 5, S6: Substrate 6, mW: milliwatt, %: percentage.

Raman spectra of SOF samples using S2 were collected using the fibre optic probe (spot size of ~100 μm). Raman spectra using S5 and S6 were obtained using the fibre optic adapted to an optical microscope. Magnification objectives of 10x, 20x 40x and 100x (spot size of 210, 105, 52 and 21 μm respectively) were evaluated using S5 and SOF containing cocaine at 20 mg/mL to determine the highest Raman signal. Analysis of cocaine using S6 was conducted applying the same optimised magnification objective as for S5.

Samples of tongue tissue containing cocaine could not be evaluated with these substrates because of the nature of the substrates, i.e. S5 and S6 could not be applied on or mixed with tissue samples.

A minimum of three replicates were collected for each analysis using S2 and six replicates using S5 and S6. The optimal number of replicates was calculated based on the intraclass coefficient (ICC) (Saha et al. 2012) of a preliminary data set (n = 26) at three concentrations (0.1, 1 and 10 mg/mL cocaine in SOF) and the Equation 2.1. ICC values higher than 0.95 were obtained for all concentrations using 3 and 6 replicates (ICC ranged 0.95 to 0.98).

3.2.5.1 *Method optimisation*

The following parameters were evaluated: (1) limit of detection (LOD) of cocaine in SOF, (2) linearity, (3) accuracy and precision. These parameters are part of the criteria recommended for the validation of quantitative Raman methods described by the United States Pharmacopeia (USP 2011).

The **LOD** was determined as the lowest concentration at which a three-to-one signal-to-noise ratio (S/N) could be obtained. The S/N was based on the peak height from the baseline noise. The **linearity** of the method for cocaine in SOF was evaluated over a concentration range of 0.1-20 mg/mL using six standards. The regression line was calculated by the method of least squares and expressed by the correlation coefficient (R^2), linearity was assessed by F-test and visual evaluation of residual plots.

Intra-day and inter-day precision and accuracy of the method was determined at a low (0.1 mg/mL), medium (0.5 mg/mL) and high (5 mg/mL) concentration. **Intra-day precision** was calculated using six (n = 6) sets of samples obtained on the same day at each concentration level and expressed as a percentage relative standard deviation (RSD). **Inter-day precision** was evaluated at each concentration level on three different days (n

= 3) and expressed as a percentage of RSD. An acceptable value of ± 20% of the reference value was used for intra-day and inter day precision (USP 2011). **Accuracy** was calculated by dividing the mean measured concentration at each level (n = 6) by the theoretical spiked concentration and expressed as a percentage of the theoretical spiked concentration. Accuracy was reported as acceptable if the measured concentration was ± 20% of the theoretical value.

3.2.6 Analysis of tissue samples - Acquisition of Raman spectra using Raman microscopy

Samples of control porcine tissue and porcine tissue containing cocaine were analysed using the DXR$_{TM}$ Raman microscope without the use of SERS substrates. Two excitation wavelengths of 532 nm and 785 nm were used for the analysis. Conditions of Raman spectroscopic measurements at 534 nm and 780 nm laser power are summarised in Table 3.3.

Table 3.3 Conditions used to analyse cocaine in porcine tongue tissue using the DXR$_{TM}$ Raman microscopy with excitation wavelength 532 nm and 785 nm.

Excitation wavelength (nm)	**Tongue Tissue**			**Parameters**			
	Tissue	Section of tissue	Mode	Laser Power (mW)	Exposure Time (s)	Objective (x)	Number of Scans
532	Control	1	1	10	5	2	6
		2	1				
		3	1				
	With cocaine	1	1	10	4	15	6
		2	1				
		3	1				
785	Control	1	1	24	20	3	6
	With cocaine	1	1	24	30	2	6
		2	1 2	24	30	2	6
		3	1 2	24	6	10	6

Control: Tissue without cocaine. With cocaine: tissue containing cocaine. x: Objective magnification.

Low magnification objectives were used for the analysis of porcine tongue tissue (532nm laser: 2x and 785 nm laser: 15x). These magnifications allowed the collection of spectra from the tissue as a whole and not the analysis of individual cells. Spectra were collected at three different sides of the sectioned tissue (Figure 3.1). Changes in concentration were assessed by measuring the intensity of the band at 1002 cm^{-1} at the various sections of the tongue.

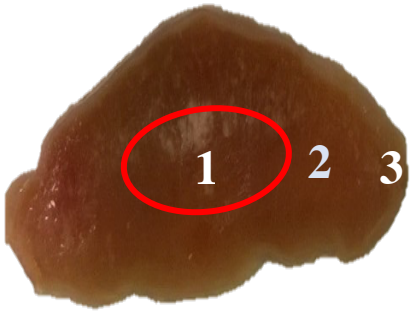


Figure 3.1 Illustration of the transverse cross-section of tongue tissue showing the sections analysed. Centre (1), off-centre (2) and edge (3).

3.2.7 Spectral data processing

All spectra were dark background corrected using the inbuilt software from the iRaman Plus instrument. Comparison between spectra was conducted by setting the baseline in the proximities of the cocaine Raman peak (1000 cm^{-1}) to zero. All spectra collected with the DXR$_{TM}$ dispersive Raman microscope were pre-processed to remove artefacts caused by background fluorescence and intensity fluctuations using a background subtraction method (polynomial 6th degree) from the OMNIC software. Band intensity at 1000 cm^{-1} of cocaine was used for qualitative analysis. Statistical analysis was conducted using the software ~IBM SPSS Version 23. Kruskal-Wallis Test H test was used for comparison of groups. Results with 2-tailed $p_s < 0.05$ were considered significant.

3.2.8 GC-MS Analysis

The amount of cocaine in porcine tissue exposed to crack cocaine was confirmed by GC-MS. Sample extraction and quantification by GC-MS was conducted using the method described by Rees et al. (2012). In summary, 1 mL of homogenised tissue was extracted using SPE cartridges equilibrated and conditioned with 2 mL methanol and 2 mL 0.1M Phosphate buffer (pH 6.0). Cartridges were washed with 2 mL deionised water, 2 mL 0.1 M HCl and 3 mL methanol. Samples were eluted using 2 mL of fresh dichloro-methane/isopropanol/ammonium hydroxide (80:17:3 v/v/v) and subsequently dried under a stream of nitrogen (≤ 40 °C). Dried samples were reconstituted in 25 µL ethyl acetate and 25 µL BSTFA and heated for 20 min at 70°C.

Extracted samples were injected into the GC-MS instrument using 2 µL injection volume. A temperature gradient was run for a total run time of 16 min: an initial temperature of 130 °C was held for 1 min, then the temperature was ramped to 240 at a rate of 30 °C/min and held for 4 min, then ramped to 260 °C at 15 °C/min and held for 2 min, then ramped to 300 °C at 60 °C/min and held for 3.33 min. The ion-trap was operated in multiple reaction monitoring (MRM) with total ion current of 10,000 and excitation amplitude of 52.5 V. Precursor ion was 182 m/z for cocaine and 185 m/z for cocaine-d_3, quantification ion was 150 m/z for cocaine and 153 m/z for cocaine-d_3. The method had a limit of quantification (LOQ) of 5 ng/g, a linear range 0.01- 1.0 mg/0.5 Kg tissue and calibration line equation $y = 0.006x + 0.00008$ with regression coefficient $R^2 = 0.999$.

3.3 RESULTS AND DISCUSSION

3.3.1 Portable Raman spectroscopy - Analysis of synthetic oral fluid samples

The substrates employed in this chapter were selected based on their availability and differences in physical characteristics. S2 contained NPs in suspension. S2 and S6, on the other hand, were solid substrates with magnetic NPs deposited on different surfaces (paper and silicon respectively). Because of their physical differences, diverse parameters were required.

Optimised values of exposure time and laser power were described in Table 3.2. These values were optimised to obtain the highest signal without saturating the detector and/or damaging the sample or substrate. Larger exposure times were required for cocaine analysis using S2 (20 seconds) compared with S2 (1 second) and S6 (10 seconds). This extended exposure time for S2 was attributed to the increased amount of energy that is required to excite NPs in suspension (Mosier-Boss 2017). The silver NPs or groups of aggregated silver NPs in suspension, were more separated or spatially dispersed from one another compared to S2 or S6. The NPs in S2 and S6 were deposited in a smaller surface area than S2. S6 for the contrary was analysed at low laser power and exposure time due to the chemical nature of this substrate (cellulose fibres) (Mosier-Boss 2017). During the development of this study, it was observed that application of laser power with energies above 80 mW resulted in the substrate's destruction.

Detection of control SOF and cocaine in SOF using the iRaman Plus 785 nm was only achieved with the use of SERS substrates (Figure 3.2 B-C). The Raman spectrum of cocaine hydrochloride (> 98.5% purity) was obtained and used for comparison, and it is

illustrated in Figure 3.2-A. The spectra of control SOF and SOF containing cocaine (20 mg/mL) with and without the use of SERS substrates (B-I) is also illustrated in this figure.

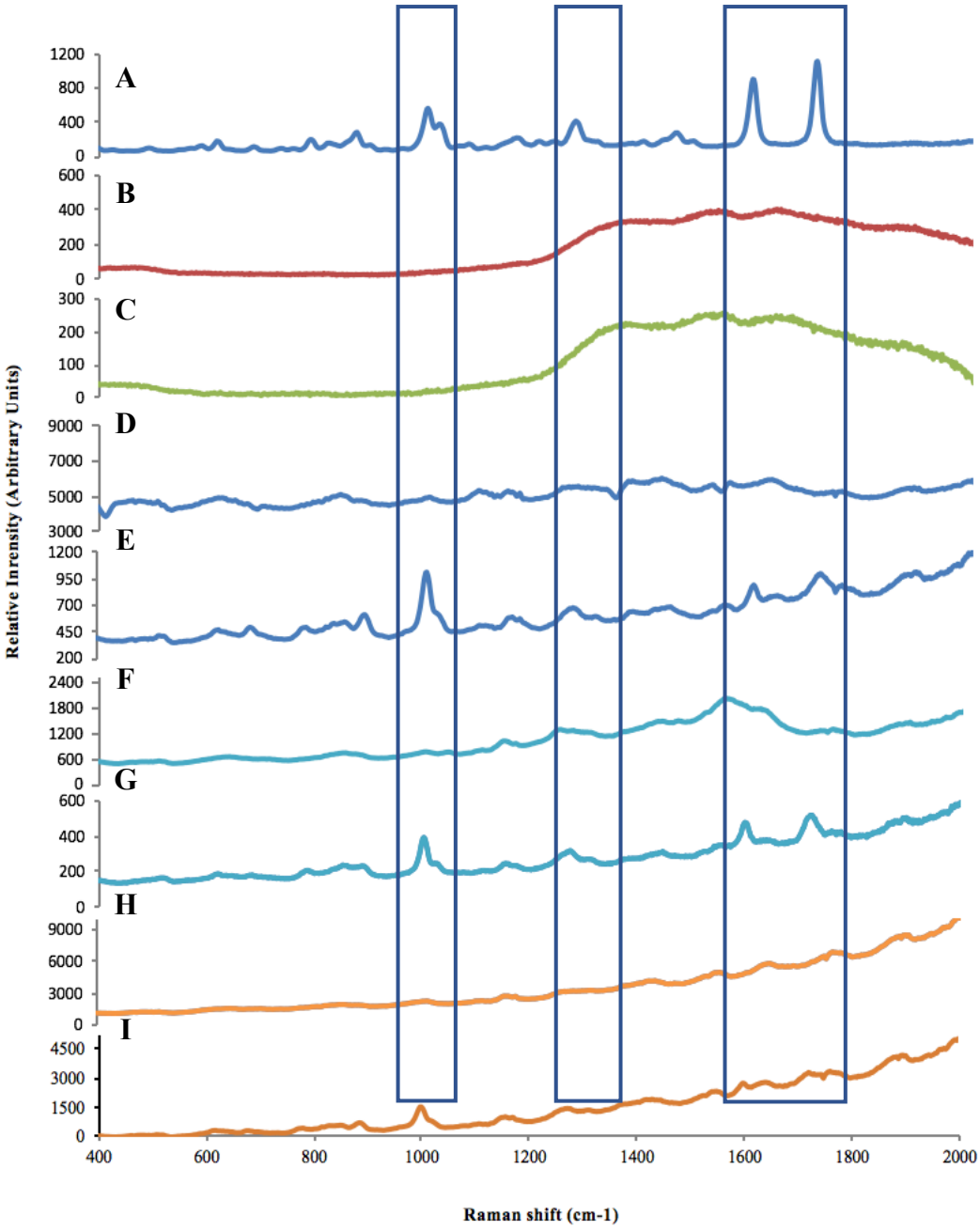


Figure 3.2 Raman spectra of (A) cocaine hydrochloride, (B) SOF, (C) SOF containing cocaine (20 mg/mL), (D) SOF using S2, (E) SOF containing cocaine (20 mg/mL) using S2, (F) SOF using S5, (G) SOF containing cocaine (20 mg/mL) using S5, (H) SOF using S6 and (I) SOF containing cocaine (20 mg/mL) using S6. Spectra obtained with iRaman and 785 nm laser. The boxes indicate the principal peaks of cocaine.

3.3.1.1 *Analysis of SOF samples using Substrate 2*

SERS spectra of SOF and SOF containing cocaine (20 mg/mL) using S2 were analysed and compared with the spectrum of cocaine hydrochloride (Figure 3.2 A) to determine characteristic peaks from the scattering of the samples. Comparison of Raman spectra of control SOF (Figure 3.2 D) and SOF containing cocaine (Figure 3.2 E) resulted in distinct differences in band positions and intensities. A very weak scattering was seen for SOF with S2. Minor peaks at 637-689, 728 and 914 cm^{-1} (-C-C- stretch) were assigned to proteins present in the SOF. The bands at 1192 and 1207 cm^{-1} (CH_2) were assigned to protein and aliphatic amino acids and the bands at1450, 1540-1589 cm^{-1} (C=C amide I) to proteins present in the SOF.

SOF containing cocaine (20 mg/mL), showed more visible bands than the control SOF. Bands at 522, 617-687, 775 cm^{-1} (-C-C- stretch) were assigned to proteins present in the SOF, the band at 887 cm^{-1} (-C-C- stretch) was assigned to the tropine ring. The scattering at 1000 cm^{-1} was characteristic of the asymmetric stretch of the phenyl ring from the cocaine. Bands at 1121 and 1215 cm^{-1} (CH_2) were assigned to the tropine ring and/or protein and aliphatic amino acids present in the SOF. The bands at 1554, 1596 cm^{-1} (C=C amide I) were from proteins found in the SOF which scattering was enhanced by the presence of the cocaine and the trigonal mode of the phenyl ring. The band at 1716 cm^{-1} (C=O stretching) was assigned to the tropine ring stretch, the symmetric and asymmetric phenyl ring breathing modes, the C-phenyl stretch, the trigonal phenyl ring breathing mode, and the ester carbonyl stretch. Appendix B shows the identification and functional group assignment for the peaks obtained with the iRaman 785 nm.

Among all bands observed in the test samples containing cocaine, the band at 1000 cm^{-1} was chosen for the quantification of cocaine because of the highest intensity and lack of interference with the response from SOF.

3.3.1.1.1 Method optimisation

SERS response for the analysis of cocaine in SOF at concentrations ranging 0.1-20 mg/mL is shown in Table 3.4. Mean SERS response and standard deviation were calculated based on three different measurements at each concentration point (0.1, 0.5, 1, 5, 10 and 20 mg/mL). Coefficients of variation for the SERS response were below 20% within the range of concentrations.

Table 3.4 Descriptive statistics of the analysis of cocaine in SOF using S2 and iRaman Plus.

Cocaine in SOF (mg/mL)	Mean (*n*=3)	SD	%RSD	Min	Max
	(Arbitrary Units)				
0.1	163	19.4	12	145	184
0.5	350	27.9	8.0	323	378
1.0	581	56.6	9.7	548	647
5.0	1211	48.7	4.0	1166	1263
10.0	1044	88.0	8.4	962	1137
20.0	1779	228	13	1571	2070

Mean: mean value of relative intensity of cocaine in SOF using S2. Min: Minimum value of relative intensity, Max: Maximum value of relative intensity, *n*: number of measurements, SD: standard deviation, %RSD: Percentage of relative standard deviation. SOF: Synthetic oral fluid.

The LOD in this study was 0.1 mg/mL cocaine in SOF using S2. This LOD was equal to the LOD obtained using a handheld Raman spectrometer (Chapter 2).

The calibration curve and residual plot (range 0.1-20 mg/mL) obtained for the quantification of cocaine in OF using S2 is shown in Figure 3.3. Figure 3.3 shows that there was a logarithmic relation between the increase in SERS response of cocaine with

the increase in concentration of cocaine in SOF. The residual plot shows a non-random pattern in the residuals, which confirmed the non-linearity of the model.

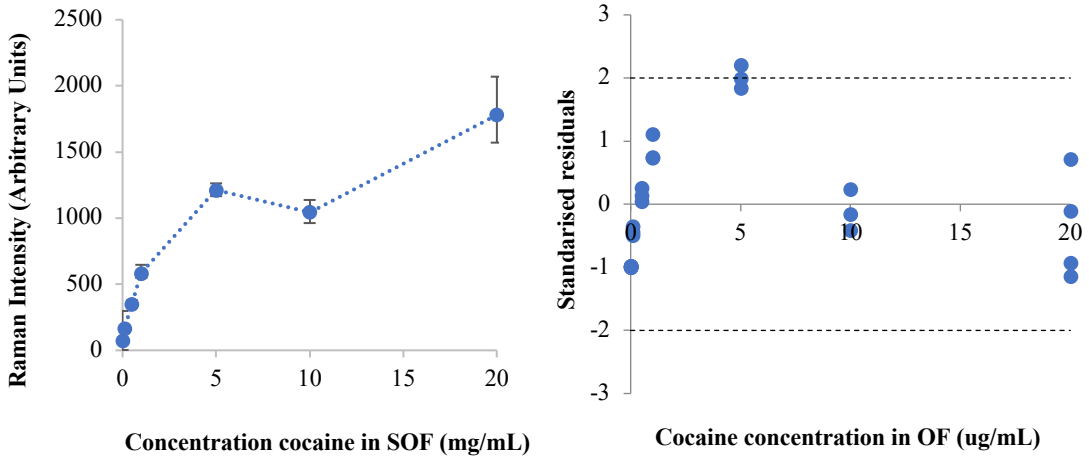


Figure 3.3 SERS calibration curve and residual plot for cocaine in SOF in a range of 0.1-20 mg/mL using S2. (Data represents mean values (n=3). Doted lines (-) represents $\pm t_{(0.95,\ np-2)}$. Data collected at Raman shift 1000 cm^{-1}using iRaman Plus 785 nm. Error bars represent the standard deviation of the data.

In order to linearise the curve, the square of the SERS response was plotted against the cocaine concentration in SOF. Figure 3.4 illustrates the linear regression for the data after some outliers were removed. A regression line with mean response values of cocaine in SOF was obtained for a concentration range of 0.1-20.0 mg/mL in SOF using six concentration points: (1) $y = 133665x - 143783$; $R^2 = 0.905$. Mean values were calculated based on a minimum of three replicates at each concentration point. Calibration lines and residual plots are shown in Figure 3.4. No random scattering of the residuals were observed with all values falling within the corresponding values of $\pm t_{(0.95\ np-2)}$, which indicated the lack of linear correlation. The F-test (α= 0.5%) indicated that the data was heterocedastic and therefore the variance around the regression line was not uniform ($p >$ 0.05). The F-test and the residual plot confirmed the non-linear regression of the model.

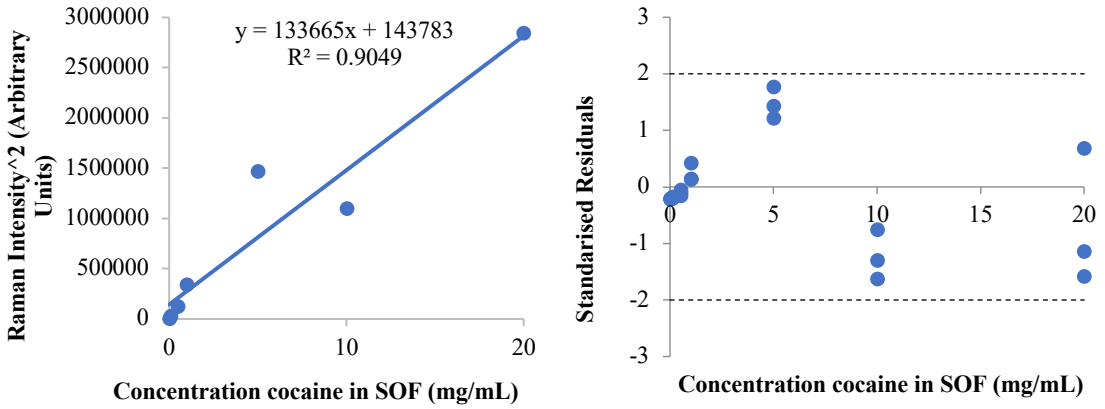


Figure 3.4 SERS calibration curve and residual plot for the square of the SERS response of cocaine in SOF in a range of 0.1-20 mg/mL using S2. (Data represents mean values (n>3). Doted lines (-) represents $\pm t_{(0.95,\ np-2)}$. Data collected at Raman shift 1000 cm^{-1}using iRaman Plus 785 nm.

The SERS response of cocaine obtained using S2 could be explained by the fact that the number of hot-spots present in the substrate were saturated and therefore the excess of cocaine molecules could not be enhanced by the NPs (Herrera et al. 2013, Schlücker 2014).

Accuracy and precision were not evaluated for S2 as the linearity of the model could not be confirmed.

3.3.1.2 *Analysis of SOF samples using Substrate 5*

Comparison of Raman spectra of control SOF (Figure 3.2 F) and SOF containing cocaine (20 mg/mL) using S5 (Figure 3.2 G) resulted in evident differences in band positions and intensities. Minimal scattering was seen for SOF with S5. Minor bands at 617 and 727 cm^{-1} (-C-C- stretch) were assigned to the aromatic ring from proteins present in the SOF. The bands at 1123 and 1221 cm^{-1} (CH_2) were assigned to protein and aliphatic amino acids and the bands at 1474, 1569 cm^{-1} (C=C amide I) to proteins present in the SOF.

Test samples of cocaine in SOF (20 mg/mL), on the other hand, showed more visible bands than the control SOF. Scattering at 522, 617, 717 cm^{-1} (-C-C- stretch) were assigned to proteins present in the SOF, the band at 887 cm^{-1} (-C-C- stretch) was assigned to the tropine ring. The scattering at 1000 cm^{-1} was characteristic of cocaine (asymmetric stretch of the phenyl ring). Bands at 1141 and 1247 cm^{-1} (CH_2) were assigned to the tropine ring and/or protein and aliphatic amino acids present in the SOF. The 1478 and 1571 cm^{-1} (C=C) bands were from proteins found in the SOF which scattering is enhanced by the presence of the cocaine and the trigonal mode of the phenyl ring respectively. And the band at 1716 cm^{-1} (C=O stretching) was assigned to the tropine ring stretch, the symmetric and asymmetric phenyl ring breathing modes, the C-phenyl stretch, the trigonal phenyl ring breathing mode, and the ester carbonyl stretch. Summary of identification and functional group assignment for S5 can be seen in Appendix B.

3.3.1.2.1 Method optimisation

Initially an evaluation of the magnification was conducted to obtain the higher SERS signal. A Kruskal-Wallis H Test showed that there was not a significant statistical difference in Raman scattering between the evaluated magnification objectives 10x, 20x, 40x and 100x ($H(3) = 6.33$, $p = 0.096$) (Figure 3.5). Although, Jonckheere's test revealed a significant trend in the data: as the number of objective magnification increased, the median of the Raman intensity decreased, $J = 27.5$, $z = -2.12$, $r = -0.51$. The intensity of the Raman signal acquired with low magnification objectives (10x and 20x) was 48% stronger (comparison of median values) than that of higher magnification (40x and 100x). These differences could be attributed to the larger amount of Raman photons being collected. Median values for the intensity of Raman spectra with the 10x and 20x showed differences of 6%. Thus, a low magnification objective of 20x was preferred. At low

magnifications, a smaller laser energy density was delivered to the surface of the sample and a larger surface area was covered.

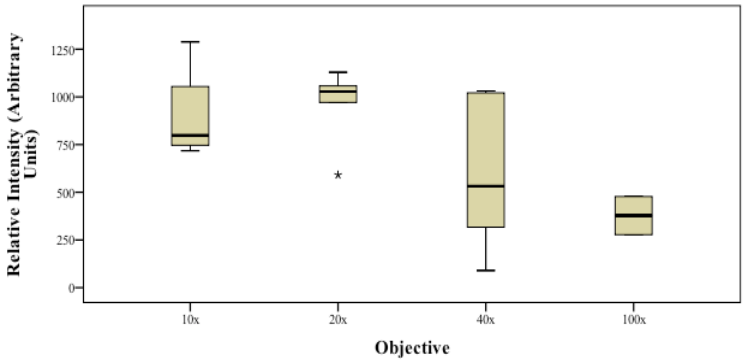


Figure 3.5 Box and whisker plot of the effect of magnification objectives on the intensity of Raman signal for the analysis of cocaine in SOF. Magnification objectives 10x, 20x, 40x and 100x. Samples at concentration of 20 mg/mL cocaine in SOF using S5. Time of interaction of 30 min. The box represents the interquartile range with the median. The whiskers extend to the 10th and 90th percentiles.

SERS response for the analysis of cocaine in SOF at concentrations ranging 0.1-20 mg/mL is shown in Table 3.5. Mean SERS response and standard deviation were calculated based on a minimum of 11 spectra per concentration point. The % RSD values were above 20% for all the concentrations.

Table 3.5 Descriptive statistics of the analysis of cocaine in SOF using S5 and iRaman Plus.

Cocaine in SOF (mg/mL)	Raman intensity (Arbitrary Units)				
	Mean (n > 10)	SD	%RSD	Min	Max
0.10	189.7	49.3	26.0	105.7	256.9
0.50	459.8	182.0	39.6	154.8	905.3
1.00	526.2	299.5	56.9	123.0	1151.5
5.00	993.4	252.2	25.4	589.1	1496.8
10.00	1540.6	695.9	45.2	596.2	2692.4
20.00	510.0	248.1	48.6	356.9	1314.5

Mean: mean value of relative intensity for cocaine in SOF using S5. Min: minimum value of relative intensity, Max: maximum value of relative intensity, n: number of measurements, SD: standard deviation, %RSD: percentage of relative standard deviation. SOF: Synthetic oral fluid.

The Figure 3.6 illustrates the SERS response of test samples against the concentration of cocaine in SOF using S5. This figure shows that the response of cocaine increased with

the increase of cocaine concentration up to a concentration of 10 mg/mL, then there was a decrease in the SERS scattering at a concentration of 20 mg/mL. Hence, the calibration curve showed a non-linear relation between the SERS response and the cocaine concentration in the SOF samples, which was confirmed by the residual plot (showing a u shape). This response could be attributed to a saturation of the hot-spots present in the substrate by the molecules of cocaine and SOF constituents, e.g. proteins and salts (Herrera et al. 2013, Schlücker 2014) at concentrations higher than 10 mg/mL. It could also be attributed to a subsequent suppression of the signal due to the excess of surrounding molecules being absorbed by the paper and on the surface of the NPs (Desai et al. 2012, Schlücker 2014).

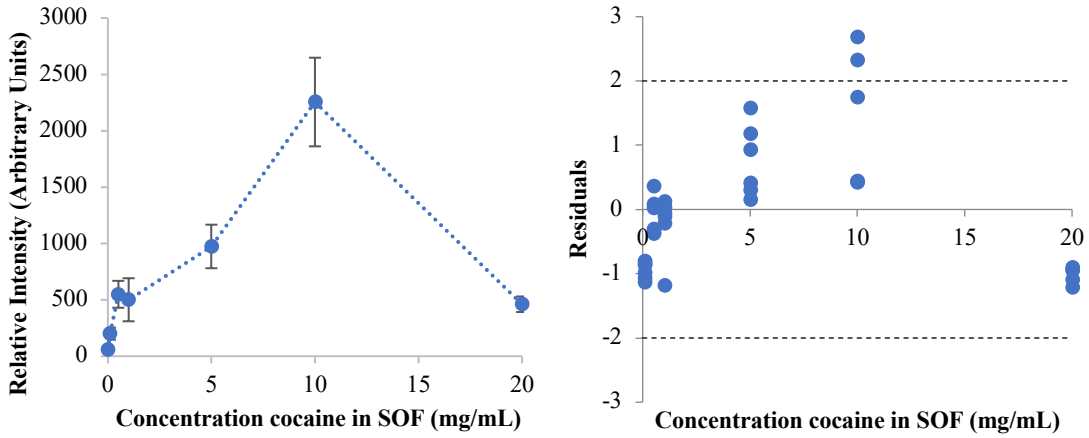


Figure 3.6 SERS calibration curve and residual plot for the SERS response of cocaine in SOF in a range of 0.1-20 mg/mL using S5. (Data represents mean values (n = 6). Doted lines (-) represents $\pm t_{(0.95,\ np-2)}$. Data collected at Raman shift 1000 cm^{-1}using iRaman Plus 785 nm. Error bars represent the standard deviation of the data.

In order to linearise the curve, the samples at concentration 20 mg/mL were excluded. Figure 3.7 illustrates the calibration curve and residual plot for the range 0.1-10 mg/mL cocaine in SOF. A linear regression was obtained using five concentration points: (1) y = 194.82x – 217.71; R^2 = 0.955. Mean values were calculated based on a minimum of six replicates at each concentration point. Random scattering of the residuals were observed

with all values falling within the corresponding values of $\pm t_{(0.95\ np-2)}$, which indicated a linear correlation. The F-test (α= 0.5%) indicated that the data was homoscedastic and therefore the variance around the regression line was uniform ($p < 0.05$). The F-test and the residual plot confirmed the linear regression of the model.

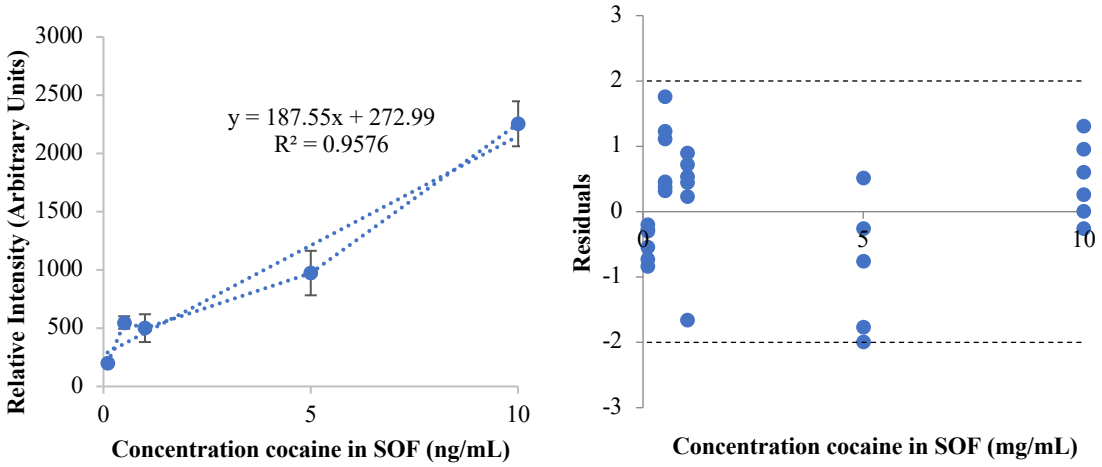


Figure 3.7 SERS calibration curve and residual plot for the square of the SERS response of cocaine in SOF in a range of 0.1-10 mg/mL using S5. (Data represents mean values (n=6). Doted lines (-) represents $\pm t_{(0.95,\ np-2)}$. Data collected at Raman shift 1000 cm^{-1}using iRaman Plus 785 nm. Error bars represent the standard deviation of the data.

The LOD achieved in this study was 0.1 mg/mL cocaine in SOF, which is the same value obtained with Substrate 2.

Intra-day precision data are summarised in Table 3.6. The intra-day precision values were > 33% for the low (0.1 mg/mL) medium (0.5 mg/mL) and high (5 mg/mL) concentration. This values were above the acceptable value of ± 20 % recommended by the United States Pharmacopeia (USP 2011). Similarly, the inter-day precision for cocaine in SOF was above the acceptable value for the low (24%) and middle (43%) concentrations. The intra-day precision at the high concentration was acceptable, with %RSD of 14%. Intra-day accuracy varied between concentrations and analysis. In general, inter-day accuracy ranged from 79-429%.

Table 3.6 Intra-day and inter-day data for analysis of cocaine in SOF using S5. Analysis conducted using a the iRaman Plus 785 nm spectrometer.

		Concentration (mg/mL)		
		0.1	**0.5**	**5**
Intra-day 1 (*n* = 6)	Mean	0.31	1.17	3.66
	SD	0.30	0.70	1.20
	SE	0.17	0.35	0.49
	% RSD	97.07	59.91	32.73
Intra-day 2 (*n* = 6)	Mean	0.47	0.49	3.20
	SD	0.31	0.34	1.32
	SE	0.18	0.14	0.76
	%RSD	65.51	68.52	41.35
Intra-day 3 (*n* = 6)	Mean	0.51	1.28	4.22
	SD	0.25	0.48	1.50
	SE	0.11	0.21	0.53
	%RSD	48.37	37.16	35.53
Inter-day (*n* = 3)	Mean	0.43	0.98	3.69
	SD	0.11	0.42	0.51
	SE	6.07	24.46	29.35
	%RSD	24.49	43.24	13.77
	Accuracy	429.27	195.94	73.86

n: Number of samples, OF: Oral fluid, SD: standard deviation, SE: Standard error, %RSD: Percentage of relative standard deviation.

Overall, the variability of the SERS response in the test samples was higher than 20%. These differences were attributed to: (1) The section of the paper analysed (number of hot-spots present in the area). (2) The differences in the surfaces of the substrates due to the deposition method, e.g. variability in the homogeneity of the NPs. (3) The chromatographic effect obtained when using paper. Paper has a capillary effect that allows the separation of substances (Yu and White 2013). The components of a test sample, e.g. cocaine and proteins, can travel across the paper at a different rate and through various sections of the paper; which could lead to differences in the number molecules absorbed on the surface of the NPs and hence to the resulted SERS scattering. (4) Inaccuracy from the Raman instrumentation, e.g. objective and detector. (5) The lack of an internal/external standard in the analysis, which could account for any variability in

surface enhancement and/or instrumental parameters, e.g. laser power, sample alignment (Loren et al. 2004).

Comparison between Substrate 2 and 5 indicated that Substrate 2 had lower variability in the measurements. Substrate 2 presented %RSD values below 20% for all concentrations evaluated whereas Substrate 5 presented values above 20% (4-13% and 26-57% respectively). The linear range obtained for substrate 2 (0.1-20 mg/mL cocaine in SOF) was higher than for Substrate 5 (0.1-10 mg/mL cocaine in SOF), although Substrate 5 presented better correlation coefficient (R^2 = 0.90 and 0.96 respectively).

3.3.1.3 *Analysis of SOF samples using Substrate 6*

SERS spectra of test samples with and without cocaine using Substrate 6 (Figure 3.2 H-I) were analysed and compared with the spectrum of cocaine (Figure 3.2A) to determine characteristic peaks from the scattering of the samples. Comparison of Raman spectra of control SOF (Figure 3.2 H) and cocaine in SOF (Figure 3.2 I) resulted in apparent differences in band position and intensities. No scattering was seen for control SOF with substrate 6. Test samples containing cocaine (20 mg/mL) showed more visible bands than the control SOF. Bands at 613, 790 cm^{-1} (-C-C- stretch) were assigned to proteins present in the SOF, the band at 895 cm^{-1} (-C-C- stretch) was assigned to the tropine ring. The scattering at 1000 cm^{-1} was characteristic of asymmetric cocaine stretch of the phenyl ring. Bands at 1164 and 1258 cm^{-1} (CH_2) were assigned to the tropine ring and/or protein and aliphatic amino acids found in the SOF. The 1445 and 1554 cm^{-1} (C=C) bands were from proteins present in the SOF which scattering is enhanced by the presence of the cocaine and the trigonal mode of the phenyl ring respectively. Summary of identification and functional group assignment for S6 can be seen in Appendix B.

3.3.1.3.1 Method optimisation

As for substrate 2 and 5, the band at 1000 cm^{-1} presented the highest Raman intensity and therefore it was chosen for the quantification of cocaine. Quantitative analysis was conducted using a minimum of six spectra per concentration point (71 SERS spectra in total). Table 3.7 shows the mean values of relative intensity along with standard deviation, %RSD and minimum and maximum values.

Table 3.7 Descriptive statistics of the analysis of cocaine in SOF using Substrate 6 and iRaman Plus 785 nm.

Cocaine in SOF (mg/mL)	Raman intensity (Arbitrary Units)				
	Mean (*n* = 6)	SD	%RSD	Min	Max
0	32	11.8	36.5	16	82
0.1	112	70	62.3	48	279
0.5	577	94	16.3	411	710
1.0	583	56	9.6	519	666
5.0	479	120	25.0	257	687
10.0	248	116	46.5	166	489
20.0	296	131	44.3	178	569

Mean: mean value of relative intensity for cocaine in SOF using S6. Min: Minimum value of relative intensity, Max: Maximum value of relative intensity, mg: Milligrams, mL: millilitres, *n*: Number of measurements, SOF: Synthetic oral fluid, SD: standard deviation, %RSD: Percentage of relative standard deviation.

The calibration curve for cocaine was evaluated over a concentration range of 0.1 – 20 mg/mL in SOF. The Figure 3.8 illustrates the SERS response of test samples using S6 against the concentration of cocaine in SOF and its residual plot. This figure shows an increase in the response of cocaine with the increase of cocaine concentration up to a concentration of 1 mg/mL. At concentrations of 5 mg/mL and above, a decrease in the response was observed (17-57%). The residual plot showed a scattering of the data within two standard deviation and a similar shape as the observed in the calibration curve. The obtained SERS response suggested that there was a saturation of the hot-spots present in

the substrate by the molecules of cocaine and SOF constituents, e.g. proteins and salts (Herrera et al. 2013, Schlücker 2014). The subsequent suppression of the signal was attributed to the excess of surrounding molecules from the SOF being absorbed on the surface of the NPs (Desai et al. 2012, Schlücker 2014).

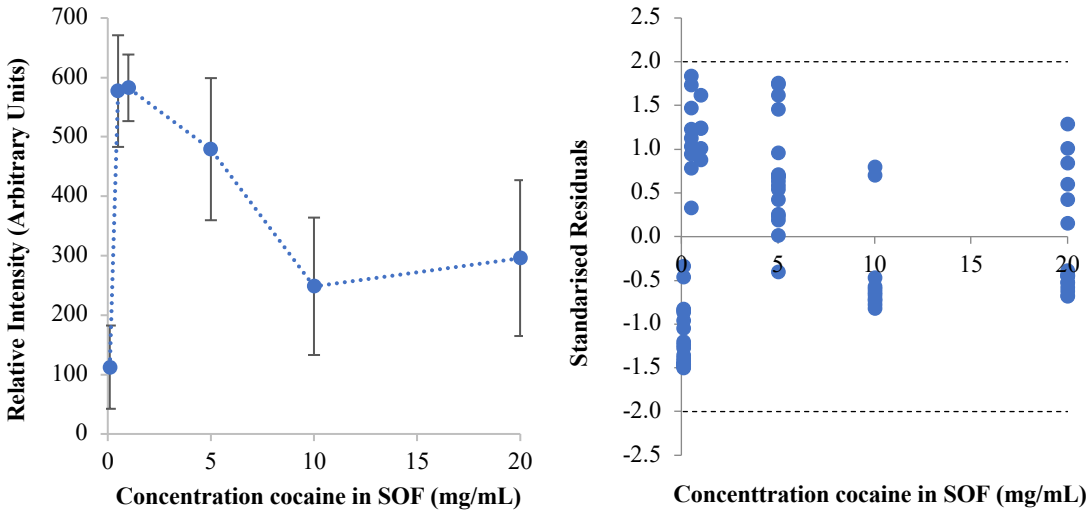


Figure 3.8 SERS calibration curve and residual plot for the SERS response of cocaine in SOF in a range of 0.1-20 mg/mL using S6. (Data represents mean values (n = 6). Doted lines (-) represents $\pm t_{(0.95,\ np-2)}$. Data collected at Raman shift 1000 cm^{-1}using iRaman Plus 785 nm. Error bars represent the standard deviation of the data.

Since a possible saturation of the hot-spots in the S6 substrate occurred at concentrations higher than 1 mg/mL, it was not possible to evaluate the linear regression of the model in the range 0.1-1 mg/mL. The USP suggest the use of six standards for the evaluation of linearity, therefore a larger number of standards need to be evaluated (USP 2011).

The LOD of this study was obtained at 0.1 mg/mL cocaine in SOF. The precision and accuracy of the method could not be evaluated because of the lack of standards available to confirm the linearity of the method (range 0.1-1 mg/mL).

The variability of the data (%RSD) obtained with Substrate 6 was higher than for Substrate 2 (Substrate 2: 4-13% and Substrate 6: 11-100%). Comparison between Substrate 5 and 6 showed no significant differences at concentrations 0.1 to 5 mg/mL ($H(3) = 3.00$, $p = 3.92$). However, at higher concentrations (10 and 20 mg/mL) Substrate 6 presented grater variability (> 93%) than the Substrate 5 (45-49%).

The potential linear range obtained for Substrate 6 (0.1-1 mg/mL cocaine in SOF) was shorted than Substrate 2 and Substrate 5 (0.1-20 mg/mL and 0.1-10 mg/mL cocaine in SOF respectively). The differences between linear range and correlation coefficients were attributed to the number of hotspots present in each of the substrates (Mosier-Boss 2017). Overall, Substrate 6 presented less variability in the results (< 20%) and Substrate 5 better linearity ($R^2 = 0.96$). The LOD, on the other hand, was the same for all substrates (0.1 mg/mL cocaine SOF).

Although detection and quantification of cocaine in SOF were obtained, the LOD obtained did not allow the detection of cocaine at physiological levels (< 3 µg/mL cocaine in SOF) or its use in *in vitro* studies. The concentration of cocaine in SOF and porcine tongue tissue has been reported to be in the range of 5-8 µg/mL at maximum (Cone 2012, Reichardt 2014).

The increase in sensitivity of SERS methods can be achieved by conducting a solid phase extraction (SPE) before the SERS analysis (Barnett and Rathmell 2015). However, SPE was not evaluated in this chapter because the time spent in the extraction procedure increases significantly the analysis, which contradicted the objectives of this research. The extraction procedure used in this chapter (a modified version of the methodology

described by Rees et al. (2012)) for the analysis of SOF samples containing cocaine based on can take up to four hours.

3.3.2 Raman microscopy - Analysis of tissue samples

Additionally to SERS analysis, i.e. use of NPs, the response of the Raman signal could be enhanced by the use of more sensitive Raman instruments (Miljković et al. 2010). Raman spectroscopy coupled with microscopy offered several advantages over handheld and portable devices because of the improvements in the design of the instrumentation (sources, optics, detectors, software). Raman microscopy has been used in the analysis of analytes at physiological levels in several biological tissues including lungs, breast, cornea, brain, oral tissue (Movasaghi et al. 2007, Freudiger et al. 2008, Matthäus et al. 2008, Kiselev et al. 2016).

3.3.2.1 *Influence of excitation wavelength*

The analysis in the Vis-Raman and NIR-Vis-Raman (excitation wavelength 532 and 785 nm respectively) demonstrated that cocaine could be detected using both excitation wavelengths. Figure 3.9 shows representative spectra of tissue containing cocaine analysed by Raman microscopy using excitation wavelengths 532 nm and 780 nm. This figure shows that bands in the VIS-Raman produced significant strengthening of the scattering than the bands in the NIR-Vis-Raman. Tongue constituents (carotenoids, haem from protein, lipids, cocaine) were more visible with the lower energy laser. These differences in scattering were attributed to the enhanced Raman scattering of intrinsic tissue chromophores like carotenoids, which have broad absorption at a maximum of 480 nm (Movasaghi et al. 2007). Resonance enhancement may occur when the incident

radiation is near the frequency of the electronic transition of tissue chromophores, which results in an enhancement (via coupling of electronic and vibrational transitions) of the intensity of inherently weak Raman bands (Synytsya et al. 2014).

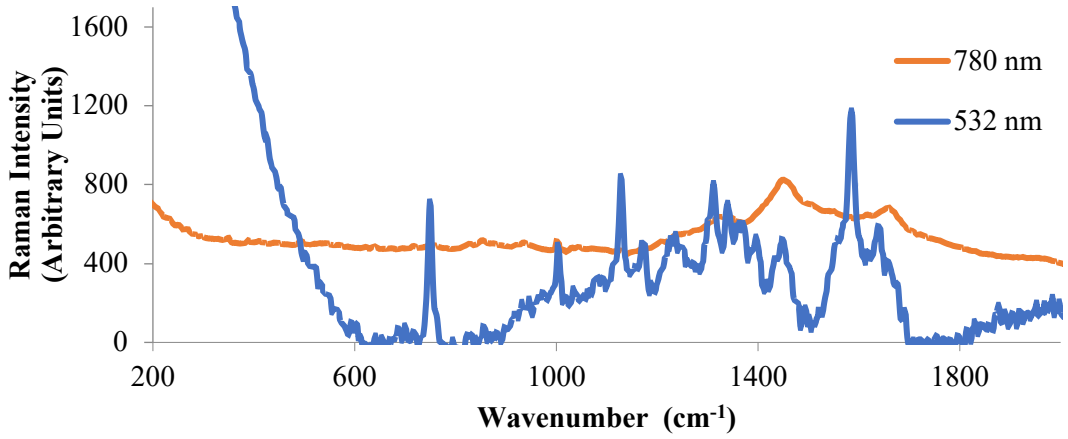


Figure 3.9 Raman spectra of tissue containing cocaine using excitation wavelengths 532 nm and 780 nm. Spectra collected with a Thermo Scientific DXR Raman microscope from section 1 of exposed tongue tissue.

The enhancement of the Raman scattering obtained with the excitation wavelength 532 nm could also be attributed to the increase in irradiated energy. This is because 532 nm excitation wavelength has higher energy than the wavelength at 780 nm and Raman scattering is proportional to the incident energy (Synytsya et al. 2014). The contribution from the incident energy was not considered significant in this study, as the time of exposure and magnification of the objective were optimised before collecting the Raman spectra at both excitation wavelengths (532 and 780 nm).

Excitation at longer wavelengths (785 cm) is frequently used for measurements of fresh tissues due to the relatively low background obtained. However, excitations at shorter wavelengths (532 cm) increase signal intensity. Unwanted auto-fluorescence and sample damage are often the main disadvantages when analysing biological tissue with shorter wavelengths. In order to obtain good spectra without sample damage a longer

scanning time was used with the excitation wavelength 785 nm (20-30 s) compared with the 532 nm (4-5 s). Under these conditions the morphology of tongue tissue remained intact, no visible changes or signs of laser damaging was observed after laser exposure.

3.3.2.2 *Analysis of tissue samples*

Test samples of tissue at a thickness of 0.02 mm showed very weak scattering with no visible bands. Tissues at 2 and 5 mm, on the other hand, showed visible bands. Hence, only the samples at a thickness of 2 mm were used for further analysis.

The chemical composition of tongue tissue comprised a distinct number of small and large molecules: amino acids, carbohydrates associated with surface epithelium, collagen component of the epithelium, globulins, lipids or muscles and proteins among others (Hand and Frank 2014). Vibrational frequencies related to different functional groups and back bond chains like proteins, nucleic acids and saccharides usually overlap specific functional groups of a particular molecule in the tissue, thus making it difficult to get accurate frequency assignments.

Comparison of Raman spectra of control tissue and tissue containing cocaine resulted in evident differences in band positions and intensities, with spectra obtained with the excitation laser 532 nm showing more resolved and intense bands. Figure 3.10 illustrates the Raman scattering of cocaine hydrochloride (> 98% purity), control tissue and tissue containing cocaine obtained with the excitation laser 532 nm. The control tissue presented minimal bands: the band at 1298 cm^{-1} was assigned to (CH_2) from protein and lipids, the band at 1439 cm^{-1} was assigned to collagen content in lipids, the bands at 1638 and 1655 cm^{-1} were assigned to (C=C amide I) from proteins and lipids and 1744 cm^{-1} to (C=O

stretching) from lipids. On the other hand, samples of tissue containing cocaine presented shifted bands at 748, 1309, 1442, 1644 and 1709 cm^{-1} (CH_2) that were assigned to protein and aliphatic amino acids. These bands are characteristic of chromophores such as collagen, myoglobin (Mb), tryptophan, etc. The presence of scattering at Raman shift of 1003 cm^{-1} was assigned to the scattering of the tropine aromatic ring from the cocaine molecule. Thus, identifying cocaine in exposed tissue only. Summary of the identification and functional group assignment for the peaks obtained with the DXR Raman microscope using excitation laser 532 nm and 785 nm can be found in Appendix B.

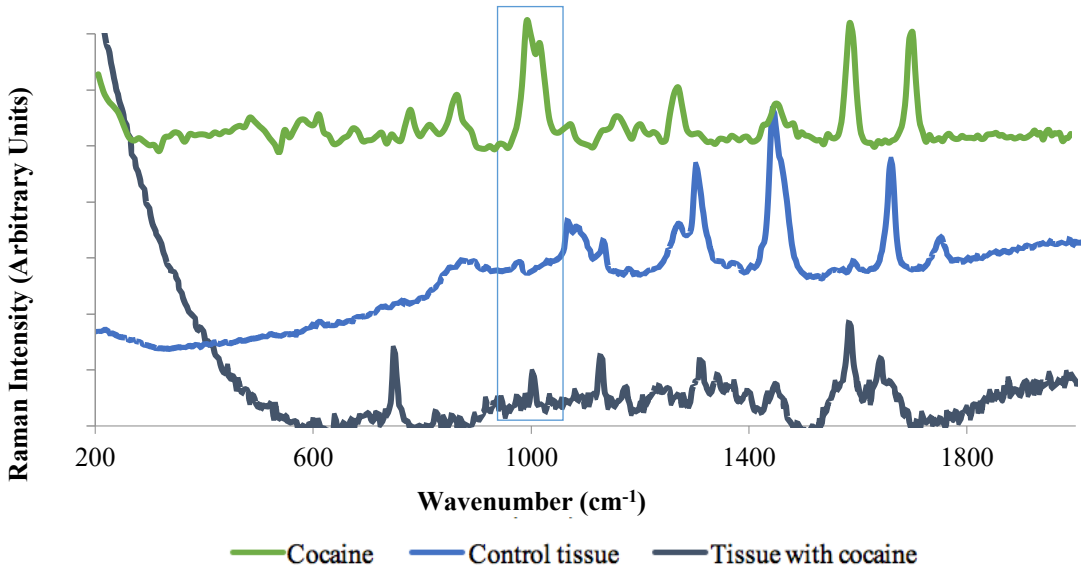


Figure 3.10 Raman spectra of cocaine hydrochloride, control tissue and tissue containing cocaine. Spectra obtained with a Thermo Scientific DRX Raman microscope and excitation wavelength 532 nm.

Although changes in the intensity and band position could be attributed to the effect that cocaine exerts in the scatter of nearest-neighbour molecules in the sample, differences in objectives (2-15x) could also have contributed to these changes (Turrell and Corset 1996). Differences in magnification objectives affected the laser energy density and therefore the Raman scattering of the sample (Sheena Mary et al. 2012, Schlücker 2014).

The analysis using the excitation laser 785 nm also showed differences in band position and intensity between the samples of control tissue and tissue containing cocaine (Figure 3.11): Control tissue presented weak bands: The band at 1122 cm^{-1} was assigned to amides present in the collagen, amino acids and proteins; the band at 1294 cm^{-1} was assigned to (CH_2) from protein and lipids, the band at 1439 cm^{-1} was assigned to collagen content in lipids, and the band at 1657 cm^{-1} was assigned to (C=C amide I) from proteins and lipids. Figure 3.11 illustrates the Raman scattering of cocaine hydrochloride (> 98% purity), control tissue and tissue containing cocaine obtained with the excitation laser 785 nm.

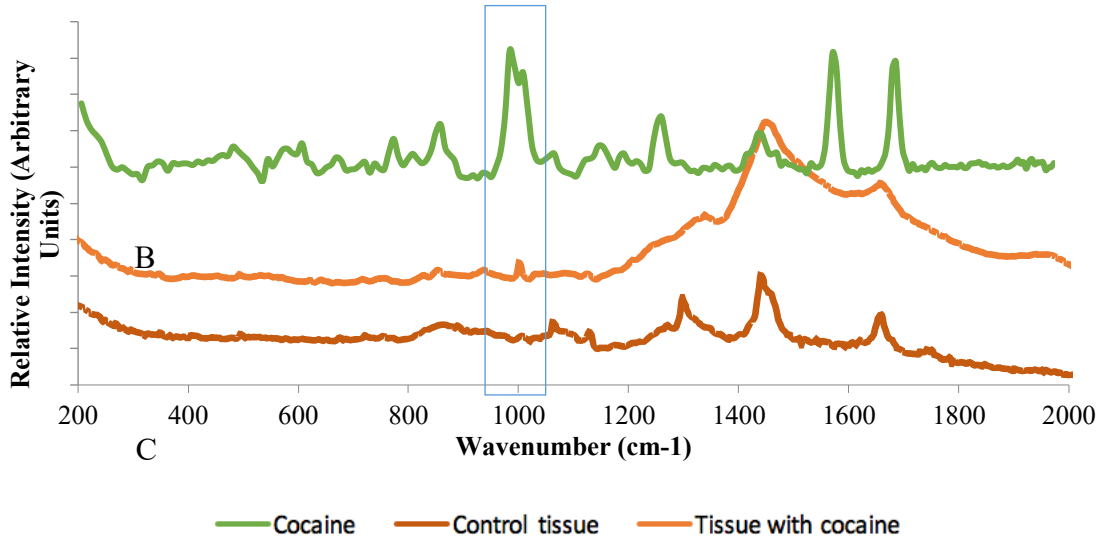


Figure 3.11 Raman spectra of control cocaine (A), control tissue (B) and tissue containing cocaine (C). Spectra obtained with a Thermo Scientific DRX Raman microscope and excitation wavelength 785 nm.

Tissue containing cocaine (Figure 3.11), on the other hand, presented fewer bands characteristic of chromophores such as collagen and myoglobin (Mb). The bands at 1340 and 1450 were assigned to collagen content in lipids, and the band at 1657 cm^{-1} was assigned to (C=C amide I) from proteins and lipids. A small band at 1003 cm^{-1} was assigned to the cocaine asymmetric stretch of the phenyl ring and therefore the presence of cocaine in tongue tissue.

3.3.2.3 *Identification of drug depots*

Variability in scattering from the tissue samples containing cocaine at the different sections of the tongue tissue is illustrated in Figure 3.12. Differences in band intensity (cocaine concentration) were seen in NIR-Vis-Raman and Vis-Raman. Analysis with the excitation wavelengths 532 nm showed a decrease in the scattering from the outside-in of the tissue (section 1 to 3, Figure 3.1). The scattering in section 2 was 8% lower than in section 1 and the scattering in section 3 was 14% lower than in section 2. Analysis conducted with the excitation wavelengths 780 nm, on the other hand, showed a different tendency. Changes in scattering intensity for section 2 was 21% higher than section 1 and section 3 was 2% lower than section 2.

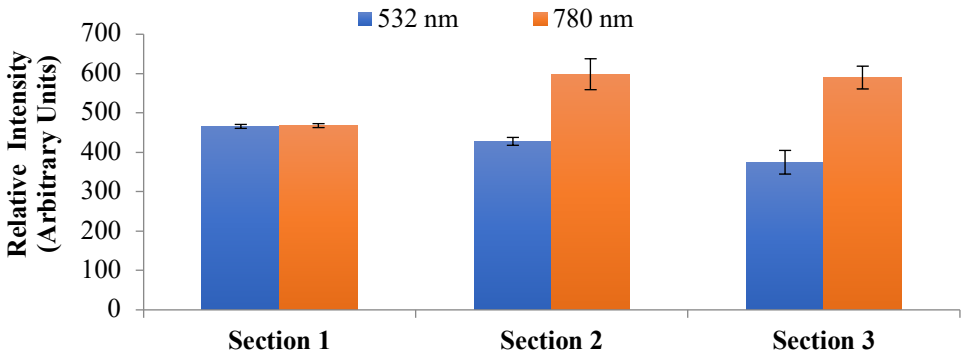


Figure 3.12 Differences in Raman scattering for test samples of tissue containing cocaine at three different sections of the tongue tissue. Spectra obtained with a Thermo Scientific DRX Raman microscope and excitation wavelength 532nm and 785 nm. Error bars indicate the standard deviation of the data.

Quantitative analysis by GC-MS of the amount of cocaine present in the tissue at the specific sections (section 1, 2 and 3) showed an increase in concentration from sections 1, 2 and 3: Section 1: 7.8 ng/g, Section 2: 24.2 ng/g and Section 3:154.1 ng/g of tissue. These results indicated that the concentration of cocaine in tissue containing cocaine decreased from the outside-in of the tissue. The trend obtained by GC-MS (i.e. increase in concentration from Section 1 to 3) was in agreement with the trend obtained using the

exposure laser 532 nm (Figure 3.12) and the results presented by Reichardt (2014), who reported that concentration of cocaine in exposed tongue tissue varied at various regions of the tissue and that concentration of cocaine decrease from the outside-in within the tissue.

During the development of this study, differences in scattering were seen within the same section (Section 1, 2 or 3). Scattering obtained at different positions in the same section differed of up to 15%. The fact that the position of the beam was not recorded when Raman analysis was conducted could have influenced the response obtained from the sample, thus explaining the variability in the response seen in Figure 3.10. The differences in Raman scattering could also be attributed to: (1) The response from a different kind of tissue, e.g. connective tissue and muscle could change based on the position of the incident light. (2) The response could be from a spot located in the proximities of the neighbour section, i.e. a spot in section 1 close to section 2. (3) The overlapping of spectral signature from different analytes, e.g. suppression of cocaine signal by the scattering of tissue constituents.

Changes in cocaine concentration (Raman scattering) were also assessed based on the colour seen in the optical images of the tissue's surface. When sections of same colour were analysed, differences in band intensity of up to 5% were found, which indicated that colour discrimination could not be used to identify regions with higher or lower drug concentration. It is important to note that at specific positions within the tissue sample containing cocaine, the Raman scattering corresponded to the fingerprint of control tissue, whereas some other areas included the scattering of the cocaine deposited in the tissue. The fact that cocaine was observed in different sides of the tissue and at different

concentrations suggested that cocaine could be specifically located within the tissue following exposure to the drug.

3.4 CONCLUSIONS

Three different SERS substrates were evaluated, for their use in the SERS analysis of cocaine in SOF (S2, S5 and S6). One homemade silver substrate and two commercial gold substrates. Selection of the substrates was based on the physical characteristics of the substrate. SERS spectra were successfully collected with all substrates for the detection of cocaine in SOF. The analysis indicated that cocaine could be detected at a LOD of 0.1 mg/mL using any of the substrates.

Quantitative analysis was possible using calibration lines within the range of 0.1-20 mg/mL for S2 ($R^2 = 0.90$) and 0.1-10 mg/mL for S5 ($R^2 = 0.96$). However, the preliminary results indicated that the precision (14-97%) and accuracy (73-429%) of the method using S5 were outside the acceptable values of ± 20% (USP 2011). The accuracy and precision of the method using S2 and S6 could not be evaluated because of the lack of linearity. Thus, a full validation (evaluation of linearity, precision, accuracy, LOD, limit of quantification, etc.) is required in order to fully evaluate the SERS methods.

The results of the analysis of cocaine in SOF using portable Raman spectroscopy proved that cocaine could be detected at levels above 0.1 mg/mL in SOF using S2, S5 or S6. However, the enhancement by these substrates (10^4) would not allow the detection of cocaine in real OF samples or its use in in-vitro studies (detection of cocaine in SOF). Levels of cocaine range between 5-8 µg/mL at maximum in OF (Cone 2012, Reichardt

2014). Therefore, a more sensitive Raman instrument or a different analytical technique should be used to obtain lower LOD.

Although all substrates allowed the detection of cocaine in SOF at LOD od 0.1 mg/mL, S2 was the substrate that provided less variability in the SERS results. Furthermore, the use of this substrate offered the advantage of more economic analysis compared with the commercial substrates, as a large volume of the substrate can be synthesised with few reagents and at atmospheric conditions.

The results presented in this chapter also concluded that cocaine in porcine tongue tissue (7-154 ng/mL) could not be detected using any of the SERS substrates and the portable iRaman spectroscope. Enhancement of the Raman signal using S5 and S6 could not be achieved due to the physical nature of the substrates (these substrates could not be mixed or applied on the surface of the tissue). Even though S2 could be applied on the surface of the tissue, the detection of cocaine in the tissue would not be achieved, as concentrations of cocaine in tissue are below the LOD of this substrate.

Raman spectroscopy coupled with microscopy provided molecular level information, enabling the investigation of drugs in porcine oral tissue. The results presented in this chapter showed that cocaine could be detected in different sections of tissue exposed to crack cocaine, using NIR-Vis-Raman and Vis Raman analysis (785 and 532 nm laser respectively). The optimal conditions for detection of cocaine in tongue tissue using the Thermo Scientific DXR Raman microscope were: excitation wavelength 532 nm and laser power 15 mW. Optimisation of parameters as the excitation wavelength was fundamental for the collection of good Raman scattering.

Evident spectral differences were seen between the tissue samples analysed in the NIR-Vis-Raman and Vis-Raman region. The bands of principal biochemical components (proteins, lipids, nucleic acids, etc.) predominated in the NIR-Vis-Raman spectra, while bands of intrinsic tissue chromophores (carotenoids, haeme) prevailed in the Vis-Raman spectra. This difference was attributed to resonance enhancement from the higher energy applied. Differences in Raman scattering were also visible at different positions of the tongue. These results indicated that cocaine was absorbed/deposited in specific areas within the tissue, thus supporting the idea of formed drug depots.

The results above mentioned indicated the presence of drug depots in tissue exposed to cocaine. However, further evaluation of the presence of drug depots could not be performed due to limitations on the availability of instrumentation. Mapping of cocaine could identify the areas where cocaine is located in tissue samples exposed to the drug and hence confirm the preliminary results obtained in this PhD thesis and the immunohistochemical results reported by Reichardt (2014).

Because of limitations with the availability of the Raman microscope, it was not possible to evaluate the detection of cocaine in SOF. However, based on the results obtained for the detection of cocaine in porcine tissue containing cocaine it can be suggested that a cocaine could be detected at physiological concentrations in SOF.

Chapter 4

VALIDATION OF LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY FOR THE ANALYSIS OF COCAINE AND COCAINE DERIVATIVES IN BUFFERED HUMAN ORAL FLUID, SYNTHETIC ORAL FLUID AND PORCINE ORAL MUCOSA

4.1 INTRODUCTION

The detection and quantification of cocaine (COC) and cocaine derivatives[1] in buffered oral fluid (BOF), synthetic oral fluid (SOF) and porcine oral mucosa tissue was required for the assessment of the release of drugs from drug depots formed in oral tissue into oral fluid (OF). The results presented in Chapter 2 and 3 demonstrated that the detection and quantification of cocaine by handheld and portable Raman spectroscopy could not be achieved for cocaine in SOF at physiological concentrations (LOD: 0.1 mg/mL for cocaine in SOF). However, it demonstrated that detection of cocaine in oral tissue could be achieved by Raman microscopy at nanogram levels. Although Raman microscopy could be used for the detection of cocaine and cocaine derivatives at physiological concentrations, it was not possible to confirm its detection and subsequent quantification in OF.

A wide range of concentrations of cocaine can be found in OF and oral tissue samples following consumption of cocaine: concentrations in the range of 0.42-1.3 μg/mL and 0.080-0.870 μg/mL cocaine were reported in SOF and homogenised tongue tissue respectively following exposure to the smoke of 200 mg crack cocaine (Reichardt 2014). Furthermore, concentrations of cocaine in OF ranging 0.014-8.6 μg/mL were reported following immediate drinking of a cup of coca tea (Reichardt 2014). In addition to Reichardt's studies, other authors have reported concentrations of cocaine in OF ranging

[1] Metabolites and other products such as anhydroecgonine methyl ester AEME (Section 1.2.3.3)

0-3 µg/mL following oral administration (chronic cocaine administration) (Kato et al. 1993; Cone 2012).

This chapter describes the development and validation of two LC-MS methods for the analysis of cocaine and cocaine derivatives in three different biological matrices: BOF, SOF and porcine oral mucosa samples. Liquid chromatography coupled to mass spectrometry (LC-MS) was selected for the quantitation of cocaine and cocaine derivatives in BOF, SOF and oral tissue because of its high sensitivity, selectivity and reliability (Drummer 2006, Bosker and Huestis 2009). Solid phase extraction (SPE) was conducted prior to the LC-MS analysis to eliminate interferents from the biological matrices, reduce the matrix effect and increase the selectivity and sensitivity of the LC-MS method (Valente et al. 2010, Rees et al. 2012).

These methods were subsequently used to evaluate the release of drugs from drug depots into OF using an *in vivo* (Chapter 6) and *in vitro* model (Chapter 7). Anhydroecgonine methyl ester (AEME), benzoylecgonine (BZE) and cocaine (COC) were evaluated in SOF and porcine oral mucosa as these analytes are the more likely to be encountered when cocaine or crack cocaine have been orally consumed (Kintz et al. 1997). BZE is the primary degradation product of cocaine (Jufer et al. 2006) and AEME is the pyrolysis product of cocaine (Lewis et al. 2004).

For the analysis of BOF the following analytes were analysed: COC, AEME, BZE, ecgonine methyl ester (EME), cocaethylene (CE) and nor-cocaine (NC). The metabolites EME, BZE and NC were included to the SOF method, to evaluate any degradation of cocaine. CE was also included in this method because this metabolite could potentially be present in collected OF, e.g. in cases where the participant had ingested alcohol

previously to the study. AEME, on the other hand, was evaluated as preliminary studies demonstrated that AEME could be an intrinsic analyte from the coca leaves. Hence, it could form drug depots in the oral cavity and its monitoring can aid in the understanding of release of drugs from drug depots.

4.1.1 AIM AND OBJECTIVES

4.1.1.1 Aim:

This chapter aimed to develop quantitative LC-MS methods for the analysis of cocaine and cocaine derivatives in SOF, BOF and porcine oral tissue.

4.1.1.2 Objectives:

- To develop and validate an LC-MS method for the quantitation of AEME, BZE and cocaine in SOF using a single quadrupole LC-MS instrument.
- To develop and validate an LC-MS method for the quantitation of AEME, BZE and cocaine in porcine oral tissue using a single quadrupole LC-MS instrument.
- To develop and validate a method for the quantitation of AEME, BZE, EME, CE, NC and cocaine in buffered OF (BOF) using a triple quadrupole LC-MS/MS instrument.

4.2 METHODS

4.2.1 Materials

The analytical standards used for the analysis on SOF and tissue: AEME and BZE were purchased from LGC Standards (Teddington, UK). Cocaine hydrochloride 10 mg (purity 99.9%) was purchased from Sigma-Aldrich (Pool, UK). Deuterated internal standards AEME-d_3, BZEd_3 and COC-d_3 were purchased at concentrations of 1 mg/mL in acetonitrile from LGC Standards (Teddington, UK).

The analytical standards used for the analysis of BOF: AEME, BZE, COC, CE, EME and NC were purchased at concentrations of 1 mg/mL in acetonitrile from Cerilliant, Sigma-Aldrich (Dorset, UK). Deuterated AEME-d_3, BZE-d_3, COC-d_3 and CE-d_3 were purchased at concentrations of 1 mg/mL in acetonitrile from Lipomed, Kinesis Ltd. (Cambridgeshire, UK).

Acetonitrile LC-MS grade, ammonia solution (33%), anhydrous disodium hydrogen orthophosphate, dichloromethane, hydrochloric acid (37%), isopropanol LC-MS grade, methanol LC-MS grade and potassium dihydrogen orthophosphate were purchased from Fisher Scientific (Loughborough, UK). Formic acid was purchased from VWR (Leicestershire, UK). Ammonium tartrate and sodium hydroxide were purchased from Sigma Aldrich (Dorset, UK).

TELOS® H-CX 130 mg 3 mL mixed-mode SPE columns were purchased from Kinesis (Cambridgeshire, UK). Oasis® mixed-mode cation exchange (MCX) micro elution plates (Waters, Manchester, UK) were donated by Alere™ Toxicology.

Concateno Certus Oral Fluid collection devices were donated by Alere™ Toxicology.

Porcine cheeks were purchased from L F B Meats, Bournemouth, UK.

4.2.2 Instrumentation

SOF and porcine oral tissue analysis was conducted using an Agilent 1200 Infinity Series LC system coupled to an Agilent Singe Quadrupole 6120 series MS system (Agilent Technologies, Waldbronn, Germany). The analysis was conducted using the electrospray ionisation source (ESI) set on positive mode. Capillary voltage was set to 4000V. Corona current was set to 4 µA. Nitrogen was used as the nebuliser gas (40 psi) heated to 200°C and drying gas flow at 11 L/min heated to 250°C. The instrument was operated in selected ion monitoring (SIM) mode. Chromatographic separation was achieved using a C18 stationary phase (column Poroshell 120 EC-C18 4.6 x 100 mm, 2.7 µm) maintained at 40°C. A column filter (in-line filter, 0.2 µm, Waters, UK) was used in front of the analytical column. Separation was achieved using gradient elution as will be discuss later in this chapter. The ChemStation software (Version A.02.) was used for system control and data acquisition. Quantitative analysis was conducted using the Quantitative Analysis MassHunter Workstation software (Version B.07.01). The analysis was conducted by the researcher at Bournemouth University.

BOF analysis samples was conducted using a LC-MS/MS system comprising of a Waters Xevo TQ MS (tandem quadrupole mass spectrometer) coupled to a Waters Acquity UPLC® system. The Analytes were separated on an Acquity UPLCTM BEH C18 column (130Å, 1.7µm, 1mm x 100mm) (Waters, Manchester, UK). Positive ESI was used and all analyses were performed in multiple reaction monitoring (MRM) mode with at least one transition (and maximum of three target ions) for each analyte. The capillary voltage was set to 4500V and desolvation temperature was 450 °C. Desolvation and cone

gas flow were 900 L/h and 50 L/h respectively. Separation was achieved using gradient elution as will be discuss later in this chapter. The MassLynx software (Version V4.1 SCN950) was used for system control, data acquisition and quantitative analysis. The analysis was conducted by the researcher at Alere Toxicology (Abingdon, UK).

4.2.3 Synthetic oral fluid preparation

Synthetic oral fluid was prepared using the Cozart biosciences protocol (2008) "Production of Synthetic Saliva" (Appendix A).

4.2.4 Tissue preparation

To prepare the oral tissue homogenates, pieces of the tissue were finely cut using surgical scissors. The sectioned tissue was subsequently weighted and a solution of 0.1M phosphate buffer saline (PBS) (pH 6) was added at a weight three times that of the tissue weight. The tissue was homogenised using a PowerGen 125 homogeniser (Fisher Scientific) at 20,000 rpm until the sample was smooth and homogeneous. Homogenised tissue was then centrifuged at 30,000 rpm for 15 minutes and the supernatant layer was removed with a manual pipette and stored at -20°C for further use.

4.2.5 Collection of control human oral fluid

Samples of control human OF were collected from five human volunteers (male and female with age range 25-35) using the Concateno Certus® collection devices. OF was collected following manufacturer recommendations and under the ethical approval granted by Bournemouth University.

The collection device comprises of an absorbent pad attached to a handle that has an indicator incorporated, and a collection tube that contains a buffer (Figure 4.1). The indicator turns blue when 1 mL of OF has been collected. To collect the samples, each participant put the Certus device(s) in the oral cavity between the inner side of the cheek and the teeth until the indicator(s) turned blue. The pad was subsequently inserted into the respectively labelled tube (part of the collection device) which contained a buffer. After the collection, all devices were stored at room temperature for 24 hours. A pool of buffered OF (BOF) was subsequently obtained.

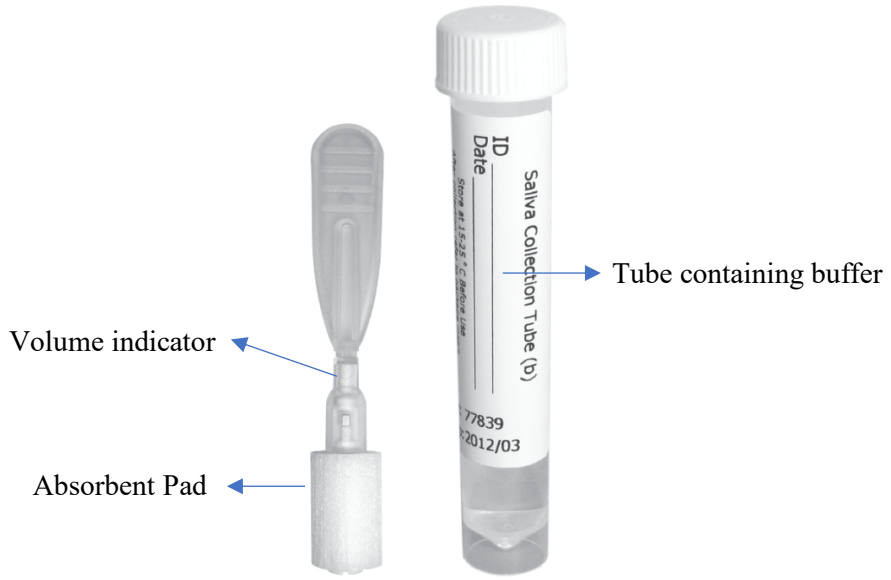


Figure 4.1 Alere™ Concateno Certus oral fluid device.

4.2.6 Solution preparation - Synthetic oral fluid and porcine oral tissue

4.2.6.1 Stock and working solutions

Initially, six stock solutions (A1, A2, A3, B1, B2, A3 and B3) containing AEME, BZE and cocaine were prepared at 1(A1/B1), 10 (A2/B2) and 100 (A3/A3) µg/mL in methanol from individual 1 mg/mL stock solutions (as purchased). The set A was used to prepare

the working solutions (5, 10, 50 and 100, 500 and 1000 ng/mL) and the set B was used to prepare the quality control (QC) standards (20, 250 and 800 ng/mL). The stock solutions were stored at -20 °C for up to three months and replaced as and when needed. Nine working solution mixtures were prepared in deionised water from the stock solutions.

4.2.6.2 Calibration and QC solutions

Calibration standards were prepared each day by fortifying 0.9 mL of drug-free SOF or homogenised tissue with the appropriate volume of working solution (100 µL), as outlined in Table 4.1.

Table 4.1 Preparation of calibration and QC solutions for the analysis of cocaine and cocaine derivatives in SOF and porcine oral mucosa.

	Level	Working solution (ng/mL)	Volume of working solution (µL)	Final volume SOF/Tissue (µL)	Calibrator/QC final concentration (ng/mL)
Calibrator	1	5	100	1000	0.5
	2	10	100	1000	1
	3	50	100	1000	5
	4	100	100	1000	10
	5	500	100	1000	50
	6	1000	100	1000	100
QC	1	20	100	1000	2
	2	200	100	1000	20
	3	800	100	1000	80

QC: Quality control; SOF: Synthetic oral fluid; Tissue: Homogenised porcine oral mucosa

4.2.6.3 Internal Standard working solution

The internal standard (IS) solution was prepared in methanol containing all deuterated analytes (AEME-d_3, BZE-d_3, COC-d_3) at a concentration of 200 ng/mL to give a final concentration of 10 ng/mL in the final samples.

4.2.6.4 Sample preparation

A 50 μL of the internal standard working solution was added to all tubes containing calibrators, QCs or samples (SOF or tissue) to obtain a final concentration of 10 ng/mL. All tubes were then vortexed for 30 seconds and transferred to clean SPE cartridges.

4.2.6.5 Blank samples

Control (drug-free) SOF or homogenate tissue was fortified with IS working solution and used as blanks. Blank matrix samples were used to monitor any carryover and to ensure that batch contamination had not occurred. For recovery determination, standard solutions were prepared at the same nominal concentration as QCs in methanol

4.2.7 Solution preparation - Buffered oral fluid

4.2.7.1 Stock and working solutions

Initially, two sets of stock solutions (A/B) were prepared. One set was used to prepare the working solutions and the second set to prepare the QC standards. Stock solution A was prepared at 20 μg/mL and B was prepared at 10 μg/mL from each analyte (AEME,

EME, BZE, COC, CE and NC) in acetonitrile from individual 1 mg/mL stock solutions (as purchased). Working solutions were prepared at concentrations of 10, 20, 200, 1000, 2500 and 5000 ng/mL. QC standards were prepared at concentrations of 15, 400 and 4000 ng/mL. The stock solutions were stored at -20 °C for up to one year and replaced as and when necessary.

4.2.7.2 Calibration and QC solutions preparation

Calibration standards were prepared by fortifying 380 µL of drug-free BOF with the appropriate volume of working solution (20 µL), as outlined in Table 4.2. The calibration standard solutions had a final concentration of 0.5, 1, 10, 50, 125 and 250 ng/mL.

Table 4.2 Preparation of calibration and QC solutions for the analysis of AEME, BZE, COC, CE, EME and NC.

	Level	Working solution (ng/mL)	Volume of Working Solution (µL)	Final Volume BOF (µL)	Final concentration (ng/mL)
Calibrator	1	10	20	400	0.5
	2	20	20	400	1
	3	200	20	400	10
	4	1000	20	400	50
	5	2500	20	400	125
	6	5000	20	400	250
QC	1	15	20	400	0.75
	2	400	20	400	20
	3	4000	20	400	200

BOF: Buffered oral fluid. QC: Quality control.

4.2.7.3 Internal Standard working solution

A mixed internal standard working internal standard solution was prepared in acetonitrile containing all deuterated analytes at a concentration of 4 μg/mL to give a final concentration of 20 ng/mL.

4.2.7.4 Sample preparation

IS working solution (20 μL) was added to all tubes containing calibrators, QCs or samples to obtain a final concentration of 20 ng/mL. Then, 200 μL of 0.1M HCl was added to all tubes. All tubes were vortexed for 30 seconds and transferred to clean SPE cartridges.

4.2.7.5 Blank samples

Control (drug-free) BOF was fortified with IS working solution and used as blanks. Then 200 μL of 0.1M HCl was added. For recovery determination, standard solutions were prepared at the same nominal concentration as QCs in methanol.

4.2.8 Solid phase extraction - Synthetic oral fluid and porcine oral tissue

SOF or tissue samples were extracted using a modification of the method described by Rees et al. (2012). The derivatisation of analytes was removed from Rees' method as this was not required for the analysis by LC-MS. TELOS® SPE cartridges were equilibrated and conditioned with successive washes of 2 mL methanol and 2 mL 0.1 M PBS (pH 6.0). Cartridges were then loaded with 1 mL of sample (calibrator, QC, blank, SOF

sample or tissue sample). Columns were washed with 2 mL deionised water, 2 mL 0.1 M hydrochloric acid and 3 mL methanol. Analytes were eluted using 2 mL of a freshly made solution of dichloromethane/isopropanol/ammonium hydroxide (80:17:3 v/v/v) solution. The excess of elution solvent was then evaporated to dryness using a gentle stream of nitrogen (≤ 30°C). Dried samples were reconstituted using 50 µL mobile phase A (aqueous solution of 10 mM ammonium formate and 0.1% formic acid).

The recovery of the SPE method was calculated using the average of three replicates at all QC levels and the Equation 4.1. Fresh unextracted standards were analysed alongside these samples.

$$Recovery\ (\%) = \frac{Peak\ Area\ Ratio\ Extracted}{Peak\ Area\ Ratio\ Unextracted} x100 \qquad \text{(Equation 4.1)}$$

4.2.9 Solid phase extraction - Buffered oral fluid samples

BOF samples were extracted using Oasis® MCX cartridges. These cartridges were available at Alere Toxicology and are routinely used for the analysis of drugs of abuse in OF. Different extraction conditions were evaluated with the Oasis® MCX cartridges in order to obtain the highest recovery of analytes. Optimisation was conducted using a low, medium and high concentration (0.75, 20 and 200 ng/mL). Table 4.3 shows the conditions evaluated for the SPE procedure. Oasis® MCX cartridges were equilibrated and conditioned with successive washes of 200 µL conditioning solution 1 (CSl) and 200 µL conditioning solution 2 (CS2). All samples were then loaded with 400 µL of sample (calibrator, QC, blank or BOF sample) mixed with 200 µL loading solution (LS). Columns were subsequently washed with 200 µL washing solution 1 (WS1) and 200 µL

washing solution 2 (WS2). Analytes were eluted using 50 µL elution solvent (ES). Following extraction of analytes, 100 µL of aqueous solution of 0.1% formic acid was added to all dwells.

Table 4.3 SPE methods evaluated for the detection of cocaine and cocaine derivatives in BOF.

	SPE steps						
Method	**Conditioning**		**Loading**	**Wash**		**Elution**	
	CS1 (200 µL)	CS2 (200 µL)	LS (200 µL)	WS1 (200 µL)	WS2 (200 µL)	ES1 (200 µL)	ES2 (200 µL)
1	MeOH	HCl	HCl:MeOH (50:50)	HCl	H_2O:ACN (70:30)	ACN	MeOH:NH_4 (97:3)
2	MeOH	HCl	HCl:MeOH (50:50)	HCl	H_2O:MeOH (70:30)	ACN	MeOH:NH_4 (97:3)
3	MeOH	HCl	HCl	HCl	H_2O:MeOH (70:30)	-	MeOH:NH_4 (97:3)
4	MeOH	HCl	PBS (pH6)	PBS (pH6)	H_2O:MeOH (70:30)	-	MeOH:NH_4 (97:3)
5	MeOH	HCl	HCl:MeOH (50:50)	HCl	H_2O:ACN (70:30)	-	MeOH:NH_4 (97:3)
6	MeOH	HCl	HCl	HCl	MeOH	-	MeOH:NH_4 (97:3)
7	MeOH	HCl	HCl	HCl	MeOH	-	MeOH:NH_4 (98:2)
8	MeOH	HCl	PBS (pH6)	PBS (pH6)	MeOH	-	MeOH:NH_4 (97:3)
9	MeOH	HCl	PBS (pH6)	PBS (pH6)	MeOH	-	MeOH:NH_4 (98:2)

ACN: Acetonitrile, NH_4: Ammonia solution, HCl: 0.1M Hydrochloric acid, CS1-2: Conditioning step 1-2, ES1-2: Elution step 1-2, LS: loading step, MeOH: Methanol, PBS: 1.0M Phosphate buffer solution pH 6, SPE: Solid phase extraction and WS1-2: Washing step 1-2.

The recovery for each SPE method was calculated using the average of three replicates at all QC levels and the Equation 4.1. Fresh unextracted standards were analysed alongside these samples.

4.2.10 LC-MS conditions - Method optimisation for synthetic oral fluid and porcine oral tissue.

Chromatographic separation was conducted using an adapted version of a previously reported method (Mack and Long 2010). The LC and MS parameters were altered several times to ensure chromatographic separation and optimum detection. The separation was achieved using aqueous 10 mM ammonium formate with 0.01% formic acid as mobile phase A and acetonitrile as mobile phase B. With flow rate 1mL/min and column temperature of 40 °C. The gradient was programmed from 7% B to 18% B over 1.5 min, then to 50% B over 2 min, then to 95% B over 0.5 min, then held for 1 min. The system was then returned to its original conditions and the column was re-equilibrated at 7% B for 3 min. The total chromatographic cycle time was 8 minutes. Chromatography was fitted to obtain retention factor k > 1 and resolution Rs > 2 for all analytes The samples were injected using a 6 µL injection volume from a 100 µL injection loop (full loop injection with loop overfill). Figure 4.2 shows the liquid chromatogram of AEME, BZE and cocaine.

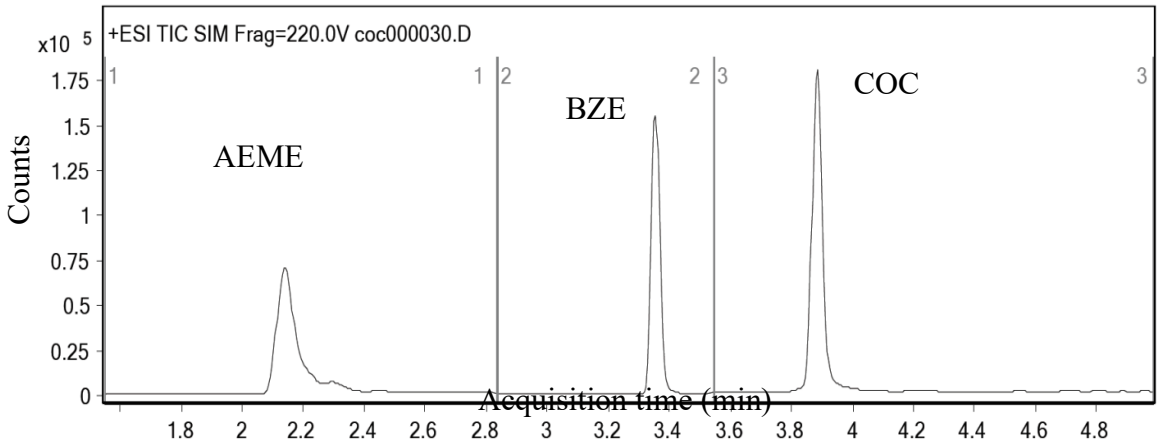


Figure 4.2 Total ion chromatogram of Anhydroecgonine methyl ester AEME (Segment 1), benzoylecgonine BZE (Segment 2) and cocaine COC (Segment 3) obtained after injection of standards at 100 ng/mL using an Agilent LC-MS single quadrupole.

Detailed method development was conducted to optimise the mass transitions for use in SIM. All drugs were run individually as standards to obtain their retention time and mass spectra. To do this, reference standards were diluted in mobile phase A to a final concentration of 100 ng/mL. Following chromatographic separation, the acquisition was split into three stages, based on the elution time. This segment separation was also used to increase the response of the ions present in the segment, as less number of ions need to be detected in a single segment of time (3-6 ions per segments instead of 16 ions). Table 4.4 presents the optimal MS parameters.

Table 4.4 Compound-specific MS (Agilent LC-MS single quadrupole) parameters used for the analysis of cocaine and cocaine derivatives in SOF and tissue.

Compound	St (min)	Rt (min)	Quantitation ion	Fragmentor Quantifier (V)	Qualifier ion	Fragmentor Qualifiers (V)	NP (psi)
AEME	1.5 - 2.5	2.13	182.1	120	91.1 122.1	220 170	40
AEME-d_3	1.5 - 2.5	2.13	185.2	120	94.1 125.2	220 170	40
BZE	2.5 - 3.5	3.36	290.1	120	168.1	220	40
BZE-d_3	2.5 - 3.5	3.36	293.3	120	171.2	170	40
COC	3.5 – 4.0	3.88	304.1	120	82.1 182.1	220 170	40
COC-d_3	3.5 – 4.0	3.88	185,1	170	85.1 307.7	220 120	40

AEME: Anhydroecgonine methyl ester, AEME-d_3: Deuterated AEME, BZE: Benzoylecgonine, BZE-d_3: Deuterated BZE, COC: Cocaine, COC-d_3: Deuterated COCAINE, St: Segment time, Rt: Retention time. NP: Nebuliser Pressure.

4.2.11 LC-MS/MS conditions - Method optimisation for buffered oral fluid

Chromatographic separation was conducted using an adapted version of the in-house routine analysis method for cocaine and metabolites used at Alere Toxicology (Abingdon, UK). The analysis was conducted at Alere Toxicology (Abingdon, UK) using a Waters Aquity instrument. The LC and MS parameters were altered several times to ensure chromatographic separation and optimum detection. Mobile phase A was an aqueous solution of 0.01% formic acid and mobile phase B was MeOH. The flow rate was 0.4

mL/min and the column temperature was 55°C. The gradient was programmed from 5% B held for 0.25 min, then raised from 5% to 60% B over 2 min, then to 80% B over 0.25 min and finally held for 0.75 min. The system was then returned to its original conditions and the column was re-equilibrated at 5% B for 1.75 min. The total chromatographic cycle time was five minutes. The samples were injected using a 5 µL injection volume. Figure 4.3 shows a typical chromatogram of EME, AEME, BZE, COC, CE and NC.

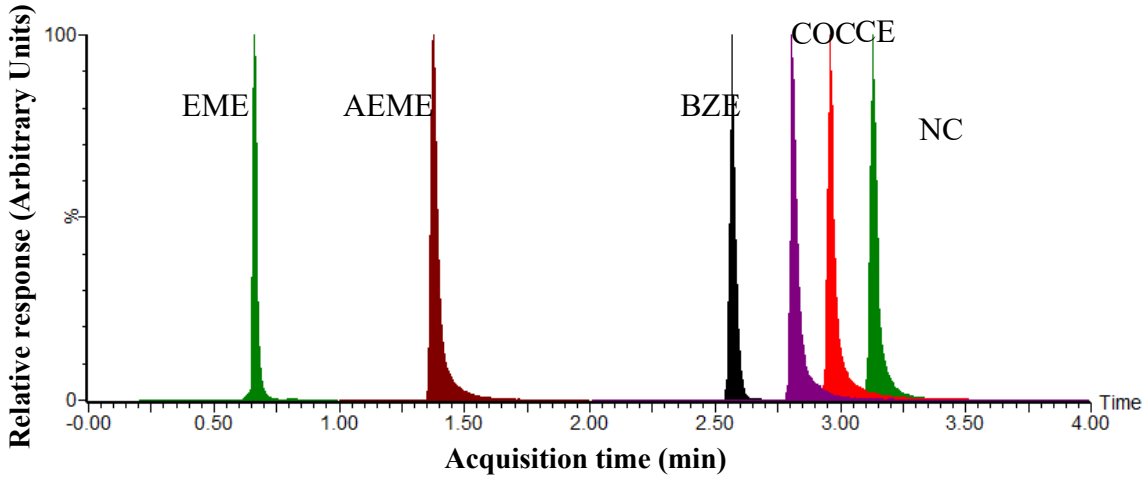


Figure 4.3 Total ion chromatograms of ecgonine methyl ester (EME), anhydroecgonine methyl ester (AEME), benzoylecgonine (BZE), cocaine (COC), cocaethylene (CE) and norcocaine (NC) obtained at 100 ng/mL using a Waters LC-MS/MS Xevo TQ.

Detailed method development was conducted to optimise the mass transitions for use in MRM. All drugs were run individually as standards to obtain their individual retention times and mass spectra. To do this, reference standards were diluted in mobile phase A (0.1% FA) to a final concentration of 100 ng/mL. Following chromatographic separation, the acquisition was split into three stages, based on the elution time. Table 4.5 presents the optimal MS parameters.

Table 4.5 Compound-specific MS (Waters LC-MS/MS Xevo TQ) parameters used for the analysis of cocaine and cocaine derivatives in BOF.

Compound	St (min)	Rt (min)	MRM Transitions (m/z)	Collision energy (V)	Corona (V)
EME	0.2-1	0.59	200.1 → 82.1 200.1 → 182.1	35	20 25
AEME	1 - 2	1.35	182.1 → 118.1 182.1 → 122.1	35	25 20
AEME-d_3	1 – 2	1.35	185.1 → 125.1	35	20
BZE	2 - 5	2.56	290.1 → 105.1 290.1 → 168.1	30	30 20
BZE-d_3	2 - 5	2.56	293.1 → 171.1	30	20
COC	2 - 5	2.81	304.1 → 105.1 304.1 → 182.1	30	30 20
COC-d_3	2 - 5	2.81	307.1 → 185.1	30	20
NC	2 - 5	2.97	290.1 → 136.1 290.1 → 168.1	35	20 25
CE	2 - 5	3.13	318.1 → 82.1 318.1 → 196.1	35	25 20
CE-d_3	2 - 5	3.13	321.1 → 199.1	35	25

AEME: Anhydroecgonine methyl ester, AEME-d_3: Deuterated AEME, BZE: Benzoylecgonine, BZE-d_3: Deuterated BZE, CE: cocaethylene, CE-d_3: Deuterated CE, COC: Cocaine, COC-d_3: Deuterated COC, NC: Nor-cocaine St: Segment time, Rt: Retention time. NP: Nebuliser Pressure.

4.2.12 Methods validation

Method validation (SOF, BOF and porcine oral tissue) were conducted in accordance with the Scientific Working Group for Forensic Toicology (SWFTOX) standard practices for method validation in forensic toxicology (SWGTOX 2013). The following parameters were used to evaluate each of the methods: accuracy; intra-assay precision and inter-assay precision; linearity; matrix effect; sensitivity; specificity and recovery.

4.2.12.1 Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The LOD for each compound was assessed by determining the lowest concentration at which a drug can be detected with a signal to noise (S/N) ratio greater than three. The LLOQ was determined as the lowest concentration with S/N ratio greater than 10 and at which accuracy (± 20%) and precision criteria (± 20%) were met.

4.2.12.2 Linearity

Calibration standards were prepared by spiking control SOF, tissue or OF as outlined in Section 4.2.6 and Section 4.2.7. Calibration lines were plotted using the peak area ratio against analyte concentration and at least six calibration points per curve. The peak to area ratio was calculated using the respectively deuterated IS, e.g. COC and COC-d_3.

The linear regression with unweighted least assumes that (1) the y-direction errors are normally distributed (Gaussian distribution), and (2) that the standard deviation of y-direction errors is the same for all x values (homoscedastic data) (Motulsky and Christopoulos 2005; Miller 1991). However, in some cases, the standard deviation of y-direction errors often increases as x increases (heteroscedastic data) and a weighted regression should be used instead. Linear regression of unweighted and weighted data was calculated by the method of least squares and expressed by the correlation coefficient (R^2), linearity was assessed by F-test and visual evaluation of residual plots. The best weighting factor was chosen according to the percentage of deviation from the nominal value and expressed as %bias.

In addition to the full validation, QC procedures were routinely conducted during batch analysis. A maximum deviation of ± 20% of the mean of the nominal QC value was considered acceptable to ensure the validity of the calibration (SWGTOX 2013).

4.2.12.3 Precision and accuracy

Within-run (n = 6) and between-run (n = 3) precision and accuracy of the method was determined by employing n = 6 of QC samples spiked at low (QC1), medium (QC2) and high (QC3) concentrations. Within-run precision was calculated using n = 6 replicates obtained on the same day and expressed as a percentage relative standard deviation (%RSD) (Equation 4.2). Between-run precision was evaluated for n = 6 sets at each QC level on three different days and expressed as a percent RSD (Equation 4.3). Accuracy was calculated by dividing the mean measured concentration at each QC level (n = 6) divided by the theoretical spiked concentration and expressed as a percentage of the theoretical spiked concentration (Equation 4.4). A maximum deviation of ± 20% of the nominal QC value was considered acceptable (SWGTOX 2013).

$$Within-run\ RSD\% = \left[\frac{Standard\ deviation\ of\ a\ single\ set\ of\ samples}{Mean\ value\ of\ a\ single\ set\ of\ samples}\right] x100 \quad \text{(Equation 4.2)}$$

$$Between-run\ RSD\% = \left[\frac{Standard\ deviation\ of\ mean\ value\ for\ each\ conc.}{Mean\ value\ for\ each\ conc.}\right] x100 \text{ (Equation 4.3)}$$

$$Accuracy\ (\%) = \frac{Actual\ value - True\ value}{True\ value} x100 \quad \text{(Equation 4.4)}$$

4.2.12.4 Carryover

Carryover was assessed by injecting blank samples following the injection of three independent calibrators at the high calibrant: concentration of 100 ng/mL for SOF and homogenate porcine tissue, and 250 ng/mL for BOF. The blank samples were subsequently examined for the presence of analytes from each previous injection.

4.2.12.5 Method selectivity – interference studies

The selectivity of the method was assessed to determine if there was any interference that can cause positive results. Interferences can be caused by endogenous (analytes present in the matrix) and exogenous compounds (other analytes present in the sample). Single ion monitoring (analysis of SOF and homogenate porcine tissue) and multiple ion monitoring (analysis of BOF) were used to ensure selectivity for the analyte of interest, even for two co-eluting analytes, e.g. COC and COC-d_3 (Ekman et al. 2009). Samples prepared in mobile phase A containing all analytes and internal standards at concentration 10 ng/mL were analysed and compared with samples prepared in each matrix.

4.2.12.6 Matrix effect – Ion suppression and ion enhancement

The matrix effect refers to the ion suppression or enhancement of an analyte by the co-eluting compounds in a biological sample, e.g. SOF, BOF or Tissue (Matuszewski et al. 2003). This effect was assessed by preparing two sets of samples made up in the mobile phase (Set 1) and in extracts of blank matrices spiked with the analyte after extraction (Set 2). Set 1 and 2 samples were spiked at low (QC1), medium (QC2) and high (QC3)

concentrations. The percentage of the matrix effect was calculated by dividing the mean peak area of the extracted samples by the mean of unextracted samples (Equation 4.5).

$$Matrix\ Effect\ (\%) = \frac{Peak\ area\ extracted\ standard}{Peak\ area\ unextracted\ standard} x100 \qquad \text{(Equation 4.5)}$$

A percentage of matrix effect greater than 100% suggested an ion enhancement and a percentage less than 100% suggest an ion suppression.

4.2.12.7 Auto-sampler stability

The auto-sampler stability was assessed to determine any variability in the concentration of analytes (peak area ratios) from samples that have undergone routine preparation after a certain period of time. The concentration of the samples may change because of their degradation when samples are not immediately analysed following the extraction procedure. Delays in the analysis can be caused by instrument failure (which may take from hours to days) and/or sample re-injection. Additionally, batch run times may take up to 24 hours depending on the number of samples.

In order to evaluate the auto-sampler stability, QCs at low (QC1), medium (Q2) and high (Q3) concentrations were analysed. The QCs were subsequently left in the auto-sampler and re-injected after 24 and 96 hours (one and four days). The recovery of each analyte was calculated using the Equation 4.6. Analytes were identified as unstable if their recovery fell out of the acceptable criteria of ± 20%.

$$Recovery\ (\%) = \frac{Final\ Peak\ Area\ Ratio}{True\ Peak\ Area\ Ratio} x100 \qquad \text{(Equation 4.6)}$$

The variation in analyte response can also monitor the stability of the analytes and internal standards themselves. Although the peak area ratio may not change over time,

the analyte and respectively internal standard can degrade in the same extent and to a concentration that cannot longer be detected.

4.3 RESULTS AND DISCUSSION

4.3.1 Solid phase extraction – Synthetic oral fluid and porcine oral tissue samples

The extraction method used for the analysis of cocaine and cocaine derivatives (AEME and BZE) from SOF and porcine oral tissue samples was based on previous studies developed by Rees et al. (2012). This method was modified to fit the analysis of analytes in a LC-MS system, e.g. there was no need for a derivatisation step. Overall recovery of the SPE method for SOF and tissue samples were 88% and 88% respectively. Recovery values for all analytes in both matrices is presented in Table 4.6.

Table 4.6 SPE recovery values of cocaine and cocaine derivatives in SOF and porcine oral tissue obtained using TELOS® cartridges.

Concentration (ng/mL)	Recovery (%)					
	SOF			TISSUE		
	AEME	BZE	COC	AEME	BZE	COC
2	95.5	97.3	98.4	98.6	99.2	99.9
20	97.0	94.9	99.2	96.3	95.6	99.5
80	97.0	96.4	99.0	98.2	103	99.6

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine and COC: Cocaine, SOF: synthetic oral fluid; Tissue: homogenised porcine oral mucosa

The results showed mean recovery values of 96, 96 and 99% for AEME, BZE and COC in SOF respectively. Mean recovery values of 98, 99, 100% were obtained for AEME, BZE and COC in porcine oral tissue. Recovery values for the deuterated analytes in SOF (10 n/mL) were 93, 90 and 107% for AEME-d_3, BZE-d_3 and COC-d_3 respectively.

The recovery in tissue were 75, 110 and 103% for AEME-d_3, BZE-d_3 and COC-d_3 respectively.

4.3.2 Solid phase extraction – Buffered oral fluid samples

The extraction method used for the analysis of cocaine and cocaine derivatives (EME, AEME, BZE, CE and NC) from BOF samples was based on a method used to analyse cocaine and metabolites at Alere toxicology. This method was modified to fit the analysis of the compounds of interest (EME, AEME, BZE, COC, CE and NC). Additionally, PBS (pH 6.0) was assessed as loading solvent because high recoveries (> 95%) were reported for the extraction of EME and other analytes in OF samples using this solution (Toennes et al. 2005).

Table 4.7 shows the peak area obtained for all the SPE method evaluated for the extraction of cocaine and cocaine derivatives (EME, AEME, BZE, CE and NC). From this table, it can be observed that the extraction of EME could not be achieved using the methods 1 to 5. Hence, these methods were discarded. Even though methods 6 to 9 were able to extract EME, only the methods 7 and 9 provided more significant peak areas for EME and the rest of analytes. The method 9 provided bigger peak areas for the extraction of EME in comparison with method 7 but the peak areas of BZE, COC, CE and NC were considerably reduced (10, 64, 69 and 82% respectively) using this method 9. As the peak area of EME was only reduced by 43% using method 7 compared with method 9, this method was used for the extraction of cocaine and cocaine derivative in BOF.

Table 4.7 Analyte response from the different SPE extraction methods obtained using Oasis® micro-extraction plates.

Analyte	Concentration (ng/mL)	SPE Extraction Method								
		Peak Area (Response/min)								
		1	2	3	4	5	6	7	8	9
AEME	1	1287	1340	151849	1441	1675	109	2650	182	3956
	10	11194	2428	160278	16322	20070	148	32657	137	53702
	50	62920	28377	168197	69520	68547	126	147006	186	224759
EME	1	ND	ND	ND	ND	ND	15	352	40	516
	10	ND	ND	ND	ND	ND	18	638	74	1725
	50	ND	ND	ND	ND	ND	2	1964	521	8514
BZE	1	1104	13862	3428	163	18	460	21914	338	2669
	10	8679	19260	31603	2569	110	594	313855	337	22462
	50	51393	276693	116146	9014	416	545	1214830	366	133342
COC	1	13023	21922	1632	20725	21356	3199	54274	2606	33229
	10	102029	43643	4038	193650	204132	3261	529737	2356	370845
	50	542366.1	455895	13826	848273	897018	2917	2525980	2604	1509470
CE	1	15115	22442	389	22968	23186	388	56701	787	36900
	10	117318	44449	343	220887	231460	598	559461	421	429495
	50	635877	481312	365	954770	1008471	356	2743653	514	1762914
NC	1	3221	16681	186	4184	4138	445	20480	496	15982
	10	26719	183626	148	40275	42066	427	195194	374	173931
	50	134727	917489	227	169780	178313	414	946733	425	753667
AEME -d_3	50	16379	51352	53433	22507	28950	84024	79179	122544	118596
BZE-d_3	50	18694	642525	9717	4857	213	786952	673331	103306	71642
COC-d_3	50	178190	982177	93590	331148	382832	948417	1034307	726091	813472
CE-d_3	50	256103	1137591	81868	403984	429283	786952	673331	103306	71642

AEME: Anhydroecgonine methyl ester, AEME-d_3: Deuterated AEME, BZE: Benzoylecgonine, BZE-d_3: Deuterated BZE, CE: cocaethylene, CE-d_3: Deuterated CE, COC: Cocaine, COC-d_3: Deuterated COC, NC: Norcocaine SPE: Solid phase extraction.

The percentage of recovery obtained using the method 7 for the extraction of all analytes is described in Table 4.8. The recovery of analytes using this method ranged between 49-100% for all analytes. Mean recoveries for AEME, EME, BZE, COC, CE and NC were 56.1, 76.7, 57.4, 89.3, 99.9 and 49.0% respectively. These results indicated that COC and CE were considerably higher than the other analytes. The differences in recovery values were attributed to changes in the recovery of corresponding IS. The mean recovery values of AEME-d_3 and BZE-d_3 were 77% and 109% respectively, compared with 52% for COC-d_3 and 51% for CE-d_3. As indicated in Equation 4.1, the percentage of recovery was calculated using values of peak area ratio and this ratio is affected by the IS response, e.g. a decrease in IS response will result in a high recovery value.

Table 4.8 SPE recovery values of cocaine and cocaine derivatives in BOF obtained using Oasis® micro-extraction plates and the SPE method 7.

Concentration (ng/mL)	Recovery (%)					
	AEME	EME	BZE	COC	CE	NC
0.75	57.2	74.7	54.2	73.2	81.8	47.2
20	51.9	82.4	45.5	64.1	82.6	42.7
200	59.2	72.8	72.5	131	135	57.1

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, CE: cocaethylene, COC: Cocaine, NC: Norcocaine.

These percentage of recoveries were considerably lower in comparison with the recoveries obtained using the TELOS multimode cartridges which ranged between 96-100%. Even though most recovery values using the Oasis® micro-elution plate were <90%, the sensitivity of the MRM mode from the MS detection allowed the detection and quantification of these analytes at nano-gram levels, as indicated below.

4.3.3 LC-MS method validation – Synthetic oral fluid and porcine oral tissue

Analyte identification was initially evaluated in total ion monitoring (TIM) under low fragmentor energy (this allowed the detection of unfragmented analytes). However, no detection was obtained due to the low concertation injected (100 ng/mL) and the low sensitivity that the scanning mode offered. Since TIM could not be used for the detection of analytes, SIM was used instead. SIM allowed the detection of single ions in a longer period of time and also the rapid switching between other selected ions resulting in reduced noise and increased sensitivity.

A maximum of three target ions and qualifier ions were obtained using the flow injection analysis (FIA) option from the ChemStation software. This option can be used to optimise the main MS parameters, such as fragmentor energy. Several fragmentor voltages were checked to find the optimum voltage that provided strong molecular ions and good relative abundance of fragment ions. To do this, voltages in a range of 0 to 300 V were set at 20 V intervals. Figure 4.4 illustrates a characteristic FIA results for the analysis of AEME (parent ion 182.1 m/z). In this figure, the highest response was obtained at fragmentor voltage of 120 V. For the detection of qualifiers ions higher fragmentor voltages were used as fragmentation of the parent ion was required, e.g. 220 V for the detection of qualifier ion 91.1 m/z. Results of the optimum MS parameters including the target ions and qualifier ions used for the detection and identification of each analyte were mentioned in the methodology section (Table 4.4).

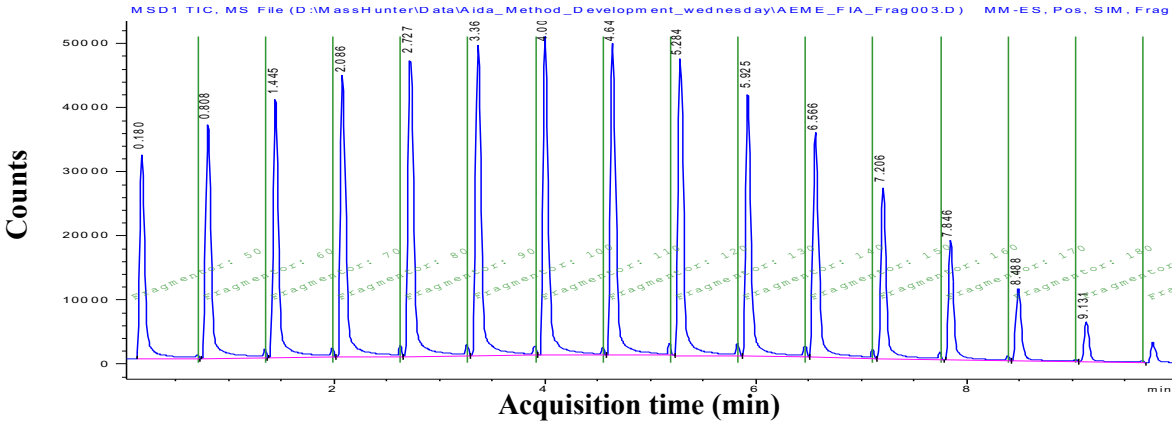


Figure 4.4 Optimisation of fragmentor voltage for the pseudo-molecular ion and fragment ion of AEME obtained using an Agilent LC-MS single quadrupole.

An illustration of the final MS spectra obtained for each analyte following optimisation is presented in Figure 4.5 Quantitation ions were selected based on the highest response as stated in Table 4.4. The quantitation ion for COC-d_3 was selected as 185.2 m/z instead of 307.7 m/z because this was the higher response obtained at the optimum fragmentor voltage.

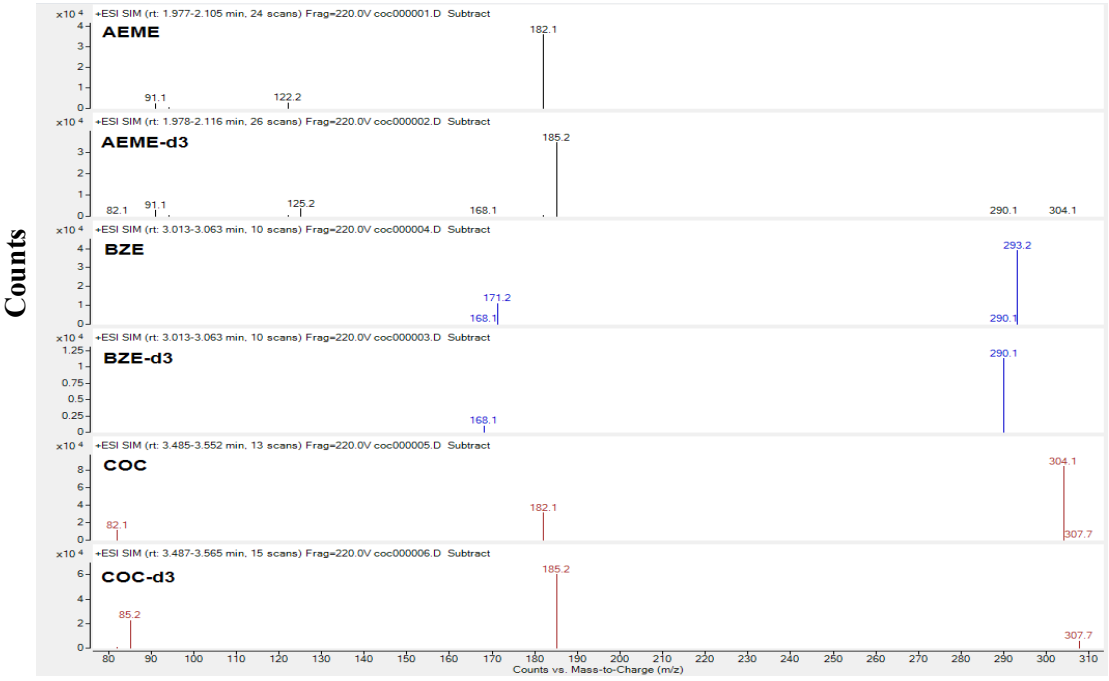


Figure 4.5 Mass Spectra (Agilent LC-MS single quadrupole) of AEME, AEME-d_3, BZE, BZE-d_3, COC and COC-d_3.

4.3.3.1 Method selectivity

No interferences were observed for AEME, BZE or COC (Figure 4.5). Additionally, no interferences were seen on any of the peaks of the non-deuterated analytes from the deuterated counterparts and vice-versa. The retention time variation for all analytes was <0.2% for all analytes (AEME: 0.20%, AEME-d_3 0.18%, BZE 0.19%, BZE-d_3 0.10%, COC 0.12% and COC-d_3 0.15%; with n = 53 for each analyte). No interferences were seen in any of the blanks of control BOF. This result indicated that there was no interference from the matrix in this method.

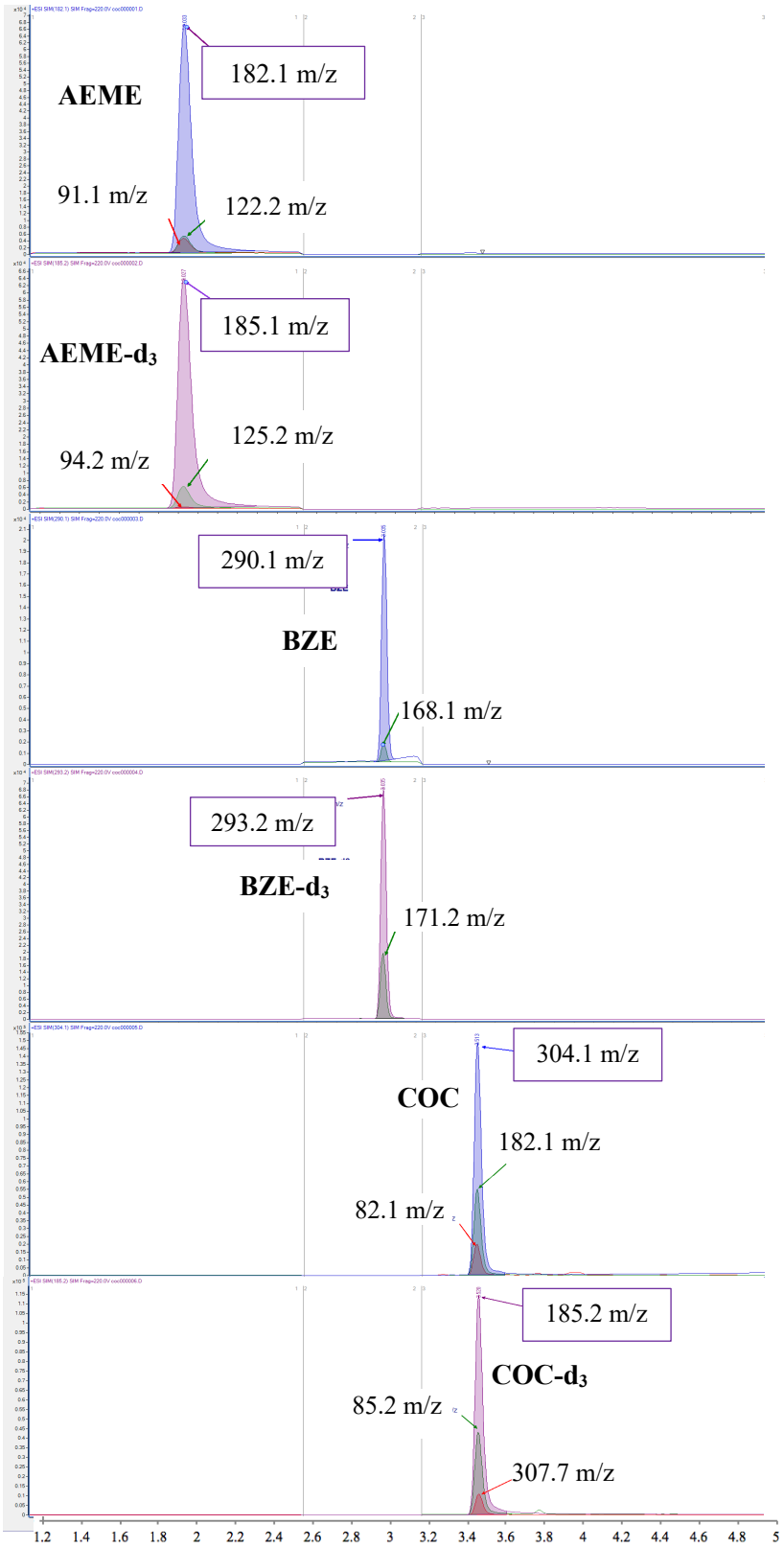


Figure 4.6 Liquid chromatograms (Waters LC-MS/MS Xevo TQ) of AEME, AEME-d_3, BZE, BZE-d_3, COC and COC-d_3 including qualifiers.

4.3.3.2 Linearity

Linearity for AEME and BZE was determined over a concentration range of 0.5-100 ng/mL in SOF or Tissue using six calibration points and six replicates per calibration point. Linearity for COC was determined over a concentration range of 1-100 ng/mL in SOF or Tissue. The residual plots (Figure 4.7) of unweighted data for all analytes in both matrices indicated that the data was heteroscedastic as the scatter of the residuals increased with the increase in concentration (Pereira da Silva et al. 2015).

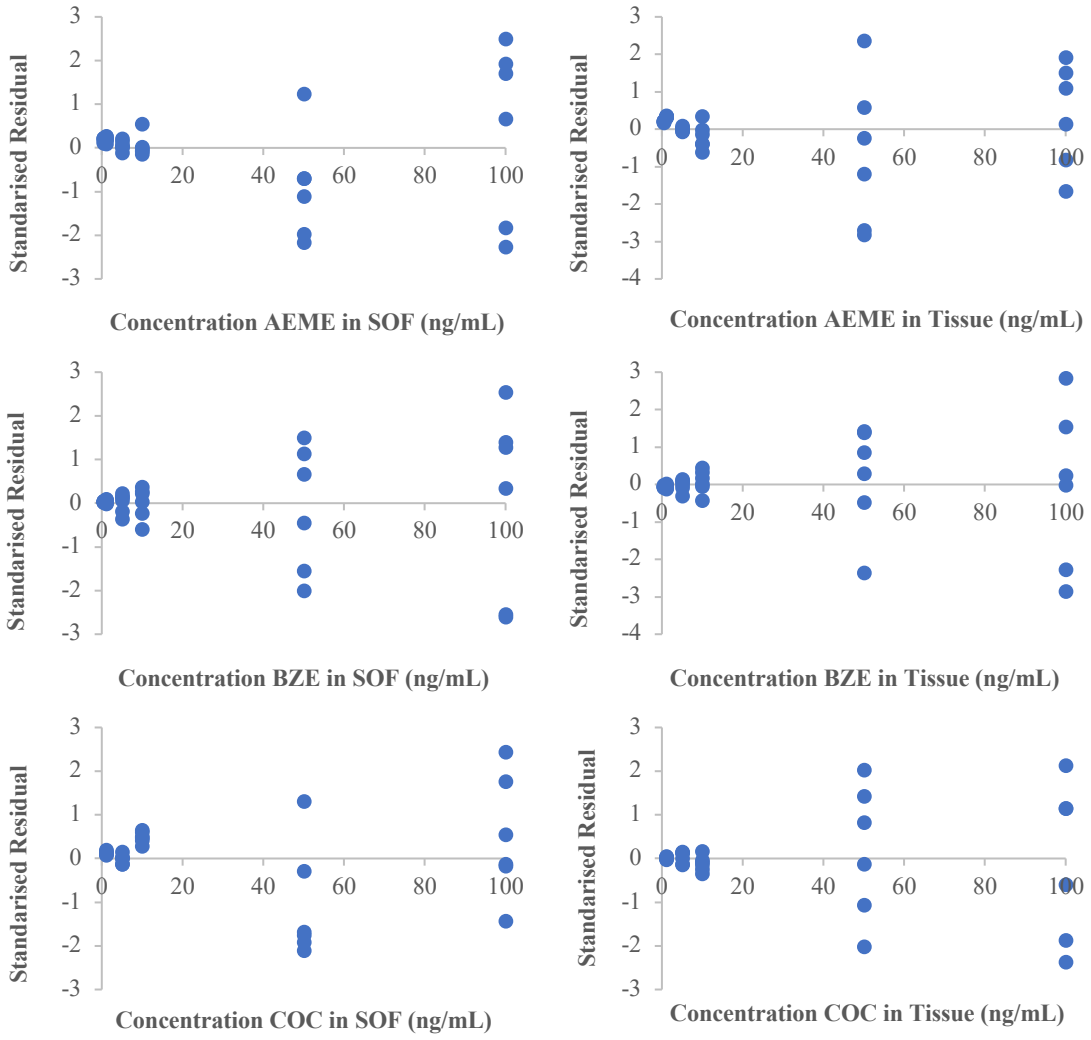


Figure 4.7 Residual plot for the analysis of anhydroecgonine methyl ester (AEME), benzoylecgonine (BZE) and cocaine (COC) in SOF and homogenate porcine tissue.

The F-Test at confidence level of 95% indicated that there were significant differences ($p < 0.05$) between the variances at the lowest and highest concentration level in all calibration lines (Table 4.9). These significant differences in variances confirmed the heteroscedasticity of the data.

Table 4.9 Results of F-Test. Comparison of the homogeneity of variances.

Analyte	Matrix	Concentration calibrant (ng/mL)	Mean PAR	F-Test	p
AEME	SOF	0.5	0.13	1893	3.48×10^{-8}
		100	10.22		
	Tissue	0.5	0.14	5020	3.04×10^{-9}
		100	10.21		
BZE	SOF	0.5	0.06	23721	6.27×10^{-11}
		100	9.81		
	Tissue	0.5	0.063	7947	9.64×10^{-10}
		100	10.27		
COC	SOF	1	0.04	1063	1.47×10^{-7}
		100	2.59		
	Tissue	1	0.04	5320	2.63×10^{-9}
		100	2.70		

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, PAR: Peak area ratio, SOF: Synthetic oral fluid, Tissue: homogenised porcine oral tissue. F-Test (5, 5, 0.95 = 5.05, $p < 0.05$).

In order to obtain a best linear unbiased relation between the concentration and the response of the analytes a weighted least squares linear regression model (1/x) was used. Weighed models are commonly used to minimise the influence of higher concentrations of the regression. Weights based on the variable x (concentration) can provide a simple approximation of the variance (Pereira da Silva et al. 2015). Results of the unweighted and weighted (1/x) linear correlation are summarised in Table 4.10. This table shows higher correlation coefficients and lower % bias (sum of the absolute % bias across the whole concentration range) for the 1/x weighting regression model than the unweighted model. The accuracy or % bias values were within ± 20% for all analytes on the weighted model. Plotting of the weighted residuals showed random scattering of variances for all analytes in both SOF and tissue matrices (Figure 4.8).

Table 4.10 Results of unweighted and weighted linear regression model for AEME, BZE and cocaine in SOF and homogenate porcine tissue.

Analyte	Matrix	Regression model	Equation	R^2	%Bias
AEME	SOF	unweighted	y = 0.1019x+0.1112	0.998	679
		1/x	y = 0.1031x+0.0786	0.998	233
	Tissue	unweighted	y = 0.1013x+0.1148	0.999	715
		1/x	y = 0.1022x+0.0892	0.999	246
BZE	SOF	unweighted	y = 0.0981x+0.0168	0.993	377
		1/x	y = 0.0984x+0.0082	0.995	291
	Tissue	unweighted	y = 0.1026x-0.0078	0.995	425
		1/x	y = 0.1020x+0.0079	0.996	254
COC	SOF	unweighted	y = 0.0261x+0.0181	0.993	424
		1/x	y = 0.2639x+0.0077	0.994	263
	Tissue	unweighted	y = 0.0269x+0.0102	0.990	233
		1/x	y = 0.0269x+0.0100	0.993	232

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, SOF: Synthetic oral fluid, Tissue: homogenised porcine oral tissue.

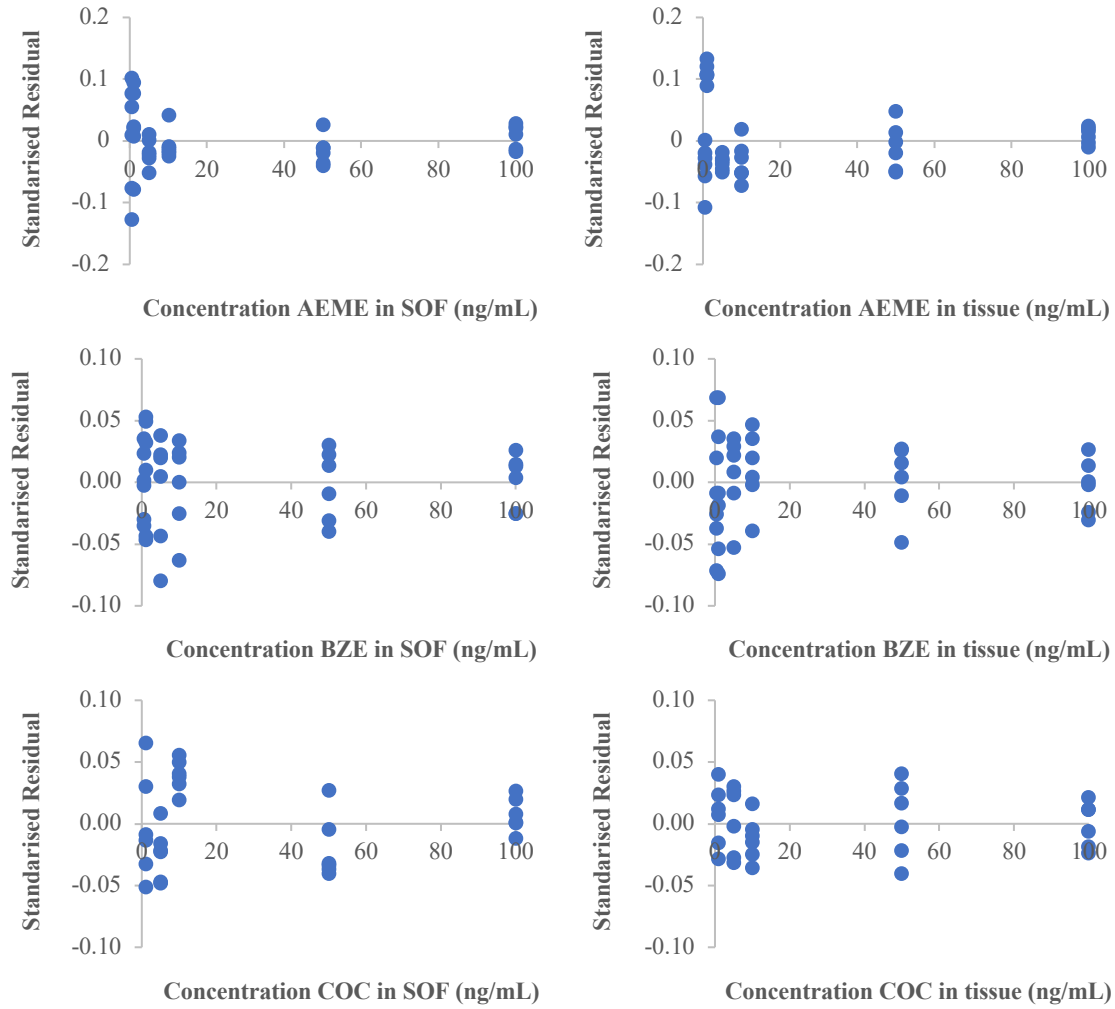


Figure 4.8 Residual plots of 1/x weighted regression model for anhydroecgonine methyl ester (AEME), benzoylecgonine (BZE) and cocaine (COC) in SOF and homogenate porcine tissue.

4.3.3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ obtained for the detection of AEME, BZE and COC in SOF are shown in Table 4.11. The results indicated that AEME and BZE could be detected (0.1 ng/mL) and quantified (0.5 ng/mL) at a lower concentration than COC (1.0 ng/mL). During the development of the method validation, it was observed the presence of a peak which corresponded to COC. Even though several washes were injected before and after every analytical run and a complete clean-up of the LC-MS system was conducted, this interfering peak could not be eliminated. Hence, the LOQ was calculated using the S/N response which included the interfering COC peak.

Table 4.11 LOD, LOQ and linearity for the analysis of cocaine and cocaine derivatives in SOF and porcine oral tissue.

Matrix	Analyte	LOD (ng/mL)	LLOQ (ng/mL)	Calibration range (ng/mL)	Calibration equation	R^2
SOF	AEME	0.1	0.5 (15.0)	0.5-100	y=0.101x-0.087	0.997
	BZE	0.1	0.5 (9.2)	0.5-100	y=0.099x-0.002	0.999
	COC	0.5	1.0 (7.0)	1.0-100	y=0.029x-0.010	0.999
TISSUE	AEME	0.1	0.5 (7.7)	0.5-100	y=0.102x-0.107	0.999
	BZE	0.1	0.5 (11.3)	0.5-100	y=0.101x-0.016	0.999
	COC	0.5	1.0 (8.3)	1.0-100	y=0.029x-0.010	0.999

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, LOD: Limit of detection, LLOQ: Low limit of quantification expressed as mean value and percent relative standard deviation (%RSD, *n*=6). SOF: Synthetic oral fluid, Tissue: homogenised porcine oral tissue.

4.3.3.4 Precision and Accuracy for synthetic oral fluid

Within-run precision data for all analytes extracted from SOF are summarised in Table 4.12. The within-run precision values were within the acceptable range of ≤ 20% (SWGTOX 2013) for the low (2 ng/mL), medium (20 ng/mL) and high (80 ng/mL) concentrations.

Table 4.12 Within-run and between-run data for analysis of AEME, BZE and COC in SOF obtained using an Agilent LC-MS single quadrupole

		Concentration (ng/mL)								
		AEME			BZE			COC		
		2	20	80	2	20	80	2	20	80
Within-run 1 (n=6)	Mean	2.01	20.8	80.0	1.96	19.7	80.0	2.00	18.2	80.6
	SD	0.08	0.34	2.28	0.05	0.55	1.40	0.13	0.78	2.57
	SE	0.03	0.14	0.93	0.02	0.22	0.57	0.05	0.32	1.05
	%RSD	4.09	1.63	2.85	2.35	2.77	1.75	6.35	4.28	3.19
	Accuracy	101	104	100	97.8	98.5	100	100	91.2	101
Within-run 2 (n=6)	Mean	1.92	20.5	80.7	1.93	19.9	79.9	1.79	17.8	78.5
	SD	0.05	0.63	3.45	0.01	0.08	0.59	0.08	0.35	0.67
	SE	0.02	0.26	1.41	0.00	0.03	0.24	0.03	0.14	0.28
	%RSD	2.53	3.09	4.27	0.40	0.40	0.73	4.60	1.94	0.86
	Accuracy	96.1	102	101	96.6	99.3	100	89.6	89.0	98.2
Within-run 3 (n=6)	Mean	1.83	20.4	81.2	1.96	19.9	80.6	1.85	18.5	79.4
	SD	0.16	0.57	2.83	0.05	0.08	1.16	0.17	0.83	0.74
	SE	0.07	0.23	1.16	0.02	0.03	0.47	0.07	0.34	0.30
	%RSD	8.95	2.79	3.49	2.48	0.40	1.44	9.06	4.51	0.93
	Accuracy	91.7	102	102	97.9	99.4	101	92.5	92.4	99.2
Between-run (n=3)	Mean	1.92	20.6	80.6	1.96	19.8	80.32	1.93	18.4	79.9
	SD	0.15	0.46	2.39	0.04	0.36	1.20	0.16	0.73	1.82
	SE	0.06	0.19	0.97	0.02	0.15	0.49	0.06	0.30	0.74
	%RSD	7.89	2.23	2.96	2.16	1.83	1.49	8.14	3.99	2.28

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, SD; standard deviation, SE: Standard error, %RSD: Percent relative standard deviation.

The within-run mean results were 1.9, 20.6 and 80.6 ng/mL for the low, medium and high AEME concentrations respectively. The mean results for BZE were 1.9, 19.8 and 80.2 ng/mL for the low, medium and high concentrations. The mean results for COC were 1.9, 18.2 and 79.5 ng/mL for the low, medium and high concentrations. Precision values were below 9.1% for all analytes at the three concentrations evaluated. The precision values obtained for AEME were within 8.9% (Low), 3.1% (Medium) and 4.3% (High). The values for BZE were within 2.5% (Low), 2.8% (Medium) and 1.8% (High). Precision values for COC were within 9.1% (Low), 4.5% (Medium) and 3.2% (High). Within-run accuracy varied between concentrations and analysis. In general, between-run accuracy was less than 9.0% for AEME, 2.8% for BZE and 9.1% for cocaine.

The between-run precision for cocaine and derivatives in SOF was below the acceptable value of ± 20% or ± 30% if the result is close to the LOQ (SWGTOX 2013). Imprecision values were below 7.9, 2.2 and 8.1% for AEME, BZE and COC respectively.

4.3.3.5 Precision and Accuracy for porcine oral tissue

Within-run precision data for all analytes extracted from porcine oral tissue are summarised in Table 4.11. The within-run precision values were within the acceptable range of ≤ 20% (<10.9%) for all analytes at the three concentrations evaluated. The within-run mean results were 2.0, 20.5 and 80.4 ng/mL for the low, medium and high AEME concentrations respectively. The mean results for BZE were 1.9, 20.1 and 81.6 ng/mL for the low, medium and high concentrations. The mean results for COC were 1.9, 19.4 and 83.3 ng/mL for the low, medium and high concentrations. The values obtained for AEME were within 5.6%, 6.7% and 3.7% of the main value at the low, medium and high concentrations respectively. The values for BZE were within 9.4% (Low), 2.4%

(Medium) and 2.2% (High). Precision values for COC were within 10.9% (Low), 5.8% (Medium) and 10.1% (High). Within-run accuracy varied between concentrations and analysis. In general, between-run accuracy was within 4.3% of the mean value for AEME, 8.6% for BZE and 8.1% for COC.

Table 4.13 Within-run and between-run data for the analysis of AEME, BZE and COC in Tissue obtained using an Agilent LC-MS single quadrupole.

		Concentration (ng/mL)								
		AEME			BZE			COC		
		2	20	80	2	20	80	2	20	80
Within-run 1 (n=6)	Mean	2.04	19.9	79.6	1.96	19.8	81.9	1.87	18.7	83.8
	SD	0.09	0.25	1.80	0.08	0.48	1.84	0.20	1.08	8.46
	SE	0.04	0.10	0.73	0.03	0.20	0.75	0.08	0.44	3.45
	%RSD	4.28	1.25	2.26	4.15	2.43	2.24	10.9	5.79	10.1
	Accuracy	102	99.6	99.4	97.9	99.1	102.4	93.6	93.7	105
Within-run 2 (n=6)	Mean	2.06	20.8	81.3	1.83	20.3	81.3	1.94	20.0	82.1
	SD	0.11	1.39	2.98	0.17	0.20	1.34	0.02	0.15	2.74
	SE	0.05	0.57	1.22	0.07	0.08	0.55	0.01	0.06	1.12
	%RSD	5.55	6.68	3.67	9.39	0.97	1.65	1.03	0.76	3.34
	Accuracy	103	104	102	91.4	102	102	96.9	100	103
Within-run 3 (n=6)	Mean	2.03	20.5	80.2	1.90	20.2	81.6	1.84	19.5	84.0
	SD	0.09	1.19	2.85	0.17	0.25	1.09	0.17	0.91	0.91
	SE	0.04	0.49	1.16	0.07	0.10	0.45	0.07	0.37	0.37
	%RSD	4.67	5.83	3.56	9.15	1.24	1.34	9.37	4.68	1.09
	Accuracy	101	102	100	95.0	101	102	91.9	97.6	105
Between-run (n=3)	Mean	2.04	20.4	80.4	1.89	20.1	81.6	1.88	19.4	83.3
	SD	0.01	0.47	0.88	0.07	0.27	0.31	0.05	0.65	1.06
	SE	0.01	0.27	0.51	0.04	0.16	0.18	0.03	0.38	0.61
	%RSD	0.71	2.31	1.10	3.44	1.35	0.38	2.68	3.37	1.27

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, SD; standard deviation, SE: Standard error, %RSD: Percent relative standard deviation.

The between-run imprecision for COC in SOF was within the acceptable range of ± 20% or ± 30% of mean values if the result is close to the LOQ (SWGTOX 2013). Precision values were within 2.3, 3.4 and 3.4% for AEME, BZE and COC respectively.

4.3.3.6 Carryover

No carryover was seen for the analysis of AEME and BZE. However, for the analysis of COC some carryover was seen. In order to eliminate this carryover, three washes were injected following the injection of high concentrations of COC in both SOF and Tissue samples. As mentioned above (Section 4.5.1.1), an interfering peak of COC was seen during the development of the analysis, therefore this could have contributed to the carryover seen during the method validation.

4.3.3.7 Matrix effect

The results of the matrix effect indicated ion suppression or ion enhancement of all analytes from samples in SOF or Tissue at the three concentrations evaluated. The matrix effect ranged from 16-134% for all the analytes in both matrices. From the results presented in Table 4.14, it was evident that ion suppression was higher at the lowest concentrations for all analytes in both SOF and tissue matrices. Except from AEME in SOF at the low concentration, which presented ion enhancement (109%). Ion enhancement was also observed for BZE in both matrices (SOF: 117%; Tissue: 134%) at the high concentration and COC in Tissue (101%) at the high concentration. Overall, the percentage of ion suppression of COC was greater than the suppression of BZE across all the concentrations. Matusewski et al. (2003) suggested that the presence of either an absolute or relative matrix effect does not necessarily suggest that a method is not valid. As long as the analyte and the internal standard (IS) exhibits the same relative matrix effect, the peak area ratio used to calculate the drug concentration should not be affected. Ion suppression or enhancement was compensated by the use of deuterated internal standards (Matuszewski et al. 2003, Bosker and Huestis 2009).

Table 4.14 Matrix effect for the analysis of cocaine and cocaine derivatives in SOF and tissue.

Concentration (ng/mL)	Matrix Effect (%)					
	SOF			Tissue		
	AEME	BZE	COC	AEME	BZE	COC
2	109	55.5	16.0	40.3	61.6	22.5
20	54.5	73.0	35.2	53.6	80.1	48.6
80	81.8	117	71.0	86.1	134	101

SOF: Synthetic oral fluid, AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine.

The differences in percentages of matrix effect could be attributed to differences in chromatographic response of each analyte and to the detection process. Even though the extraction process eliminates most interferent compounds in the samples, some compounds from the matrix could still be present in the extract. These compounds of the matrices could decrease the affinity of the stationary phase for the analyte, therefore decreasing the response of the analyte (De Sousa et al. 2012). Additionally, interferent ions could lead to changes in the ionization of the analyte and consequently on their final response. Interferent ions can compete with the analyte ions for ejection from the droplet during the desolvation process (ESI mechanism) resulting in ion enhancement or suppression of the analyte (Particle Sciences 2009).

4.3.3.8 Auto-sampler stability

The results of recovery for the auto-sampler stability are presented in Table 4.15. The recovery values for all analytes in SOF and tissue were within the acceptable criteria of ± 20% (ranging 96-108%) during a period of four days. The mean recovery values obtained for AEME, BZE and COC in SOF were 102.6±0.9%, 99.8±1.2% and 101.3±0.7% respectively. Similarly, the mean recovery in tissue was 100.3±1.4%, 102.0±0.7% and 102.4±1.4% for AEME, BZE and COC. The results presented in this

section confirm that samples in SOF and tissue can be analysed or re-injected within four days following SPE extraction without any significant sample degradation. The small differences in the percentage of recovery were attributed to two factors: (1) The accuracy of the method e.g. peak areas was within a ±20% accuracy values. (2) A slight concentration of the samples produced by the evaporation of the reconstituted solvent over the time of sample preparation and their analysis/re-injection, i.e. evaporation from such small volumes (50 µL) could lead to significant concentration of the samples.

Table 4.15 Recovery values of the auto-sampler stability for the analysis of cocaine and cocaine derivatives in SOF and tissue.

Day	Concentration (ng/mL)	Recovery (%)					
		SOF			Tissue		
		AEME	BZE	COC	AEME	BZE	COC
1	2	98.8	96.3	103	99.1	102	99.6
	20	101	99.1	101	97.9	100	104
	80	103	97.0	99.8	96.1	101	100
4	2	104	100	98.9	103	101	108
	20	105	103	101	100	105	101
	80	104	104	103	106	102	102

SOF: Synthetic oral fluid, AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine.

4.3.4 LC-MS method validation – Buffered oral fluid

Analyte identification was evaluated in multiple reaction monitoring (MRM). Suitable product ions were chosen from the reference method as indicated in Table 4.5. Maximum sensitivity was accomplished by varying the voltage in the collision cell and corona needle.

Several collision and corona voltages were checked to find the optimum voltage that provided the selection of the ion transition which produced the highest response. To do

this, collision voltages in a range of 10 to 40 V were set at 5 V intervals. Cone voltages were set in a range 0 to 60 V at 5 V intervals. Results of the optimum MS parameter including the ion transitions used for the detection and identification of each analyte were mentioned in the methodology section (Table 4.5).

4.3.4.1 Method selectivity

The injection of individual analytes did not show any interfering peaks for EME, AEME, COC and CE. For the contrary, some interfering peaks were seen in the detection of BZE and NC. However, these interfering peaks did not have any influence on the response of the analyte of interest as interfering peaks had a resolution higher than two. An illustration of the chromatograms for all analytes is represented in Figure 4.10. No interference was seen on the peaks of the non-deuterated analytes from the deuterated counterparts and vice-versa. Additionally, no interferences were seen in any of the blanks of control BOF. These results indicated that there was no interference from the matrix in this method.

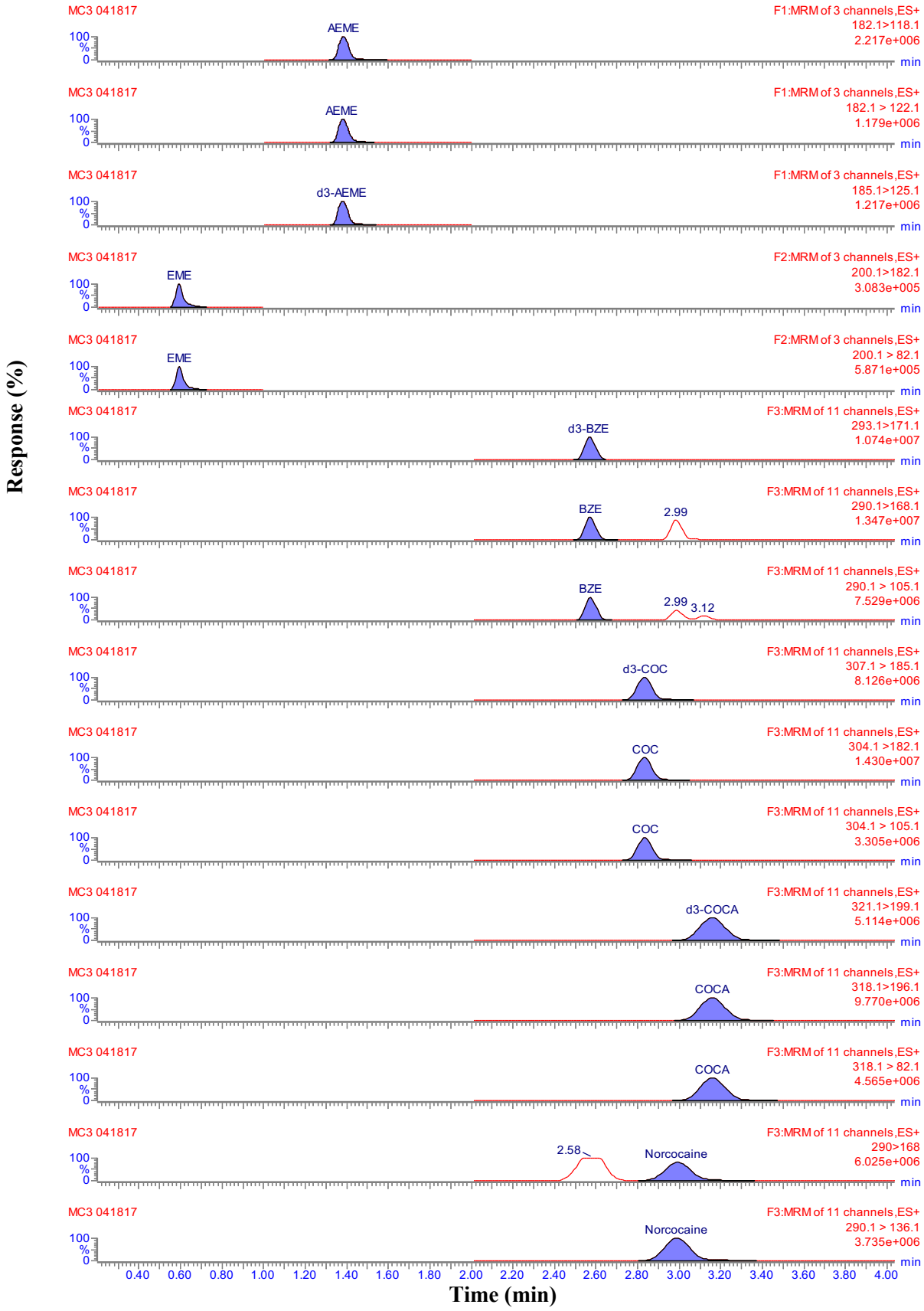


Figure 4.9 Chromatograms of AEME, AEME-d_3, EME, BZE, BZE-d_3, COC, COC-d_3, CE, CE-d_3 and NC at concentration of 10 ng/mL in OF and obtained using a Waters LC-MS/MS Xevo TQ.

The retention time variation for all analytes was < 3% (0.30% for AEME, 0.0% for AEME-d_3, 2.63% for EME, 0.19% for BZE 0.06% for BZE-d_3, 0.15% for COC, 0.09% for COC-d_3, 0.84% for CE and 0.15% for CE-d_3 and 0.65 for NC; with n = 130 for each analyte).

An increase in the response of EME was observed when a mixture of analytes (AEME, BZE, COC, CE and NC) was analysed. This enhancement was further evaluated as discussed next.

4.3.4.2 EME response

During method validation, it was observed that the response of EME when it was analysed as a mixture (in combination with the other analytes) increased significantly than when it was analysed as an independent analyte. In order to evaluate this discrepancy, two set of samples (A and B) were prepared and subsequently compared based on their EME response. The set of samples A contained EME in BOF at concentrations ranging 0.5-250 ng/mL. The set of samples B contained a mixture of AEME, BZE, COC, CE and NC in BOF at concentrations ranging 0.5-250 ng/mL of each analyte.

Table 4.16 shows the EME response obtained for the set of samples A and B. The response was reported as mean (n = 6) peak area and mean peak area ratio for the analysis of EME in BOF. The results indicated that EME was present in the set of samples B as well as for the set of samples A. Furthermore, the mean EME peak area response was 160% (120 – 230%) higher for the set of samples B than the set of samples A. The peak area ratio was 180% (120-280%). These high responses obtained for B could have been

the result of the degradation of the COC stock (Kiszka et al. 2000). Based on the fact that stock solutions and working solutions were prepared using the whole ampule of analytical standard (as purchased), it was not possible to evaluate whether or not EME was present as a result of degradation or as an impurity from any of the other analytes. Analysis of independent analytes (AEME, BZE, COC, CE and NC) from a different batch (including deuterated ISs) was conducted for detection of EME, but no response was seen in any of the analytes. Additionally, all certificates of analysis were checked for EME impurities, but then again, no impurities were reported. Since the intrinsic amount of EME present in the set of samples B was contributing to the final response of EME and the variation in the EME response (%RSD) was higher than 20% over the range of concentrations (28% and 33% for PA and PAR respectively) the validation of EME in BOF was conducted separately.

Table 4.16 Variability in peak area and peak area ratio for EME response.

Concentration in BOF (ng/mL)	Response of EME in sample A		Response of EME in sample B		Percentage of EME in B	
	PA	PAR	PA	PAR	PA	PAR
0.5	12	0.003	150	0.037	120	120
1	19	0.005	311	0.070	160	140
10	128	0.032	2916	0.921	230	280
50	643	0.165	12914	3.331	200	200
125	1631	0.387	22154	6.280	140	160
250	3624	0.927	41828	14.02	120	150

A: Set of samples containing EME: ecgonine methyl ester. B: Set of samples conteining AEME (Anhydroecgonine methyl ester), BZE (Benzoylecgonine), CE (Cocaethylene), COC (Cocaine) and NC (Nor-cocaine). PA: Peak area. PAR: Peak area ratio. Ratio: EME to Mix analytes ratio

4.3.4.3 Linearity

Linearity for all analytes (AEME, BZE, CE, COC and NC) was determined over a concentration range of 0.5-250 ng/mL in BOF using six calibration points and six replicates per calibration point. Linearity for EME was further evaluated in a larger range of 250 – 10000 ng/mL EME in BOF. This range was extended in order to be able to quantify the amount of EME present in the mixture of analytes (AEME, BZE, COC, CE and NC).The residual plots (Figure 4.10) of unweighted data for all analytes indicated that the data was heteroscedastic as the scatter of the residuals increased with the increase in concentration (Pereira da Silva et al. 2015).

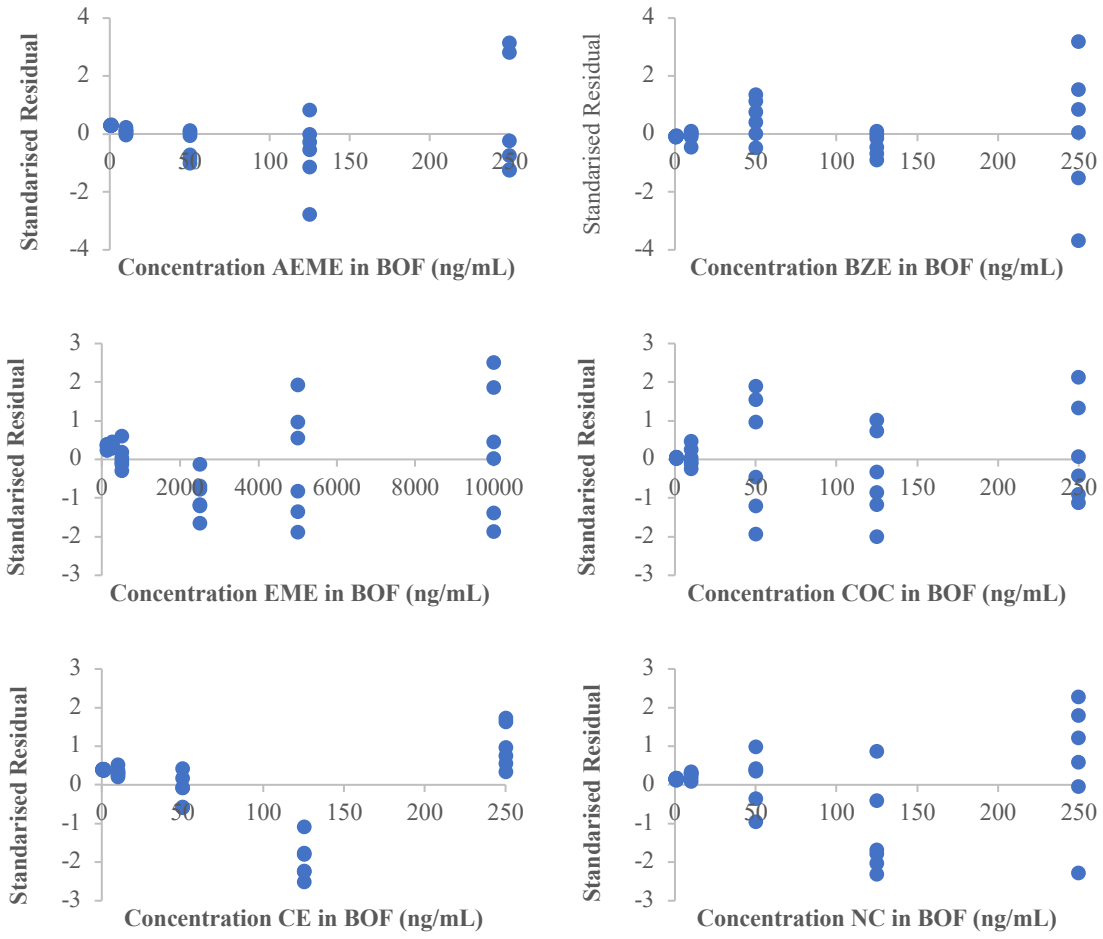


Figure 4.10 Residual plot for the analysis of anhydroecgonine methyl ester (AEME), benzoylecgonine (BZE) and cocaine (COC) in SOF and homogenate porcine tissue.

The F-Test at confidence level of 95% indicated that there were significant differences ($p < 0.05$) between the variances at the lowest and highest concentration level in all calibration lines (Table 4.17). These significant differences in variances confirmed the heteroscedasticity of the data.

Table 4.17 Results of F-Test. Comparison of the homogeneity of variances.

Analyte	Concentration calibrant (ng/mL)	Mean PAR	F-Test	p
AEME	0.5	0.35	326685	$8.90x10^{-14}$
	250	181.13		
EME	125	0.63	708.7	$4.04x10^{-7}$
	10000	36.45		
BZE	0.5	0.24	147419	$6.51x10^{-13}$
	250	170.08		
COC	0.5	0.54	5676	$2.24x10^{-9}$
	250	154.25		
CE	0.5	0.47	18024	$1.24x10^{-10}$
	250	162.36		
NC	0.5	0.23	85767	$2.52x10^{-12}$
	250	130.77		

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, PAR: Peak area ratio, SOF: Synthetic oral fluid, Tissue: homogenised porcine oral tissue. F-Test (5, 5, 0.95 = 5.05, $p < 0.05$).

In order to obtain a best linear unbiased relation between the concentration and the response of the analytes a weighted least squares linear regression model (1/x) was used. Results of the unweighted and weighted (1/x) linear correlation are summarised in Table 4.18. This table shows higher correlation coefficients and lower % bias (sum of the absolute % bias across the whole concentration range) for the 1/x weighting regression model than the unweighted model. The accuracy or % bias values were within ± 20% for all analytes on the weighted model. Plotting of the weighted residuals showed random scattering of variances for all analytes in BOF (Figure 4.12). An example of the calibration lines for all analytes in BOF obtained in this validation are presented in Appendix C.

Table 4.18 Results of unweighted and weighted linear regression model for AEME, BZE, EME, COC, CE and NC in BOF.

Analyte	Regression model	Equation	R^2	%Bias
AEME	unweighted	y = 0.7264x+1.0918	0.996	2864
	1/x	y = 0.7414x+0.0004	0.997	198
EME	unweighted	y = 0.0036x+0.2497	0.999	190
	1/x	y = 0.0036x+0.1838	0.999	83
BZE	unweighted	y = 0.6857x-0.8089	0.986	2005
	1/x	y = 0.6766x-0.1477	0.998	404
COC	unweighted	y = 0.6181x-0.0164	0.999	140
	1/x	y = 0.6188x-0.0832	0.999	98
CE	unweighted	y = 0.6606x+2.0200	0.993	5398
	1/x	y = 0.6866x+0.1353	0.995	233
NC	unweighted	y = 0.5268x+0.2523	0.998	1102
	1/x	y = 0.5308x-0.0426	0.999	172

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, SOF: Synthetic oral fluid, Tissue: homogenised porcine oral tissue.

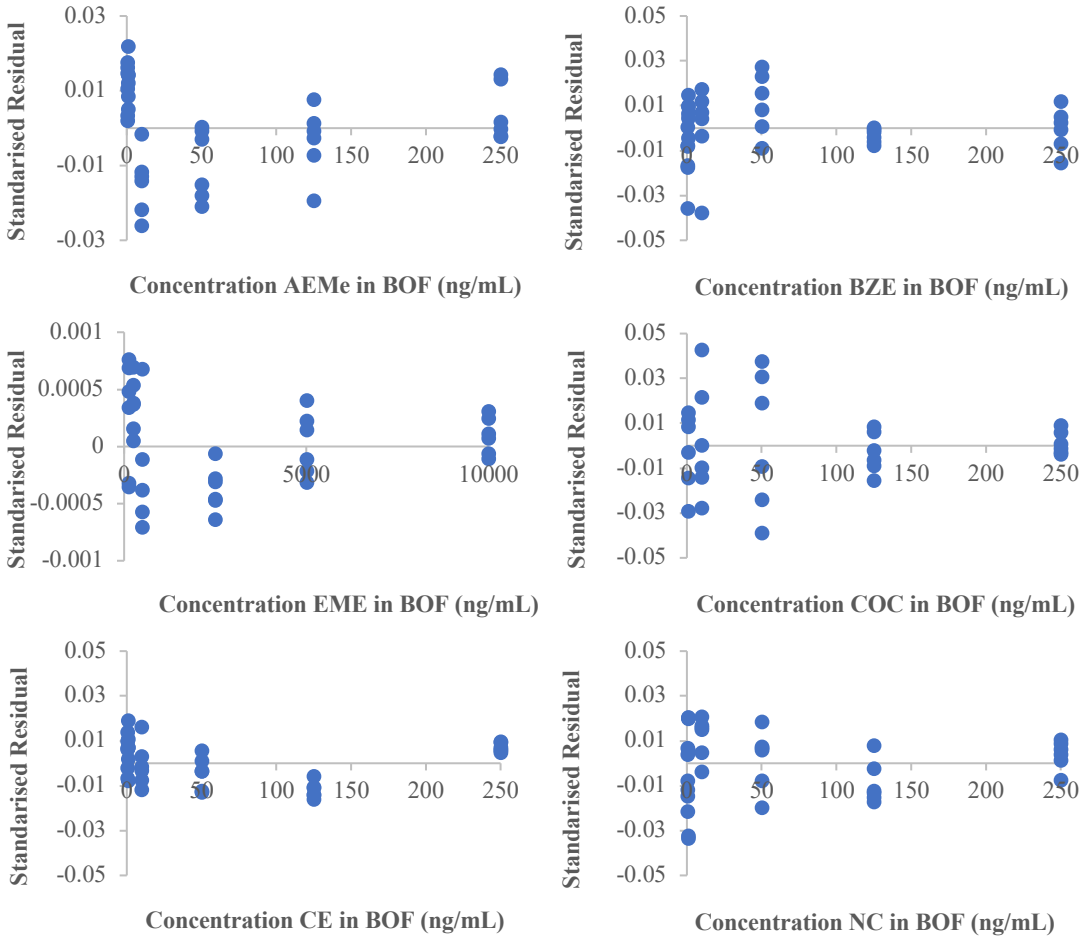


Figure 4.11 Residual plots of 1/x weighted regression model for anhydroecgonine methyl ester (AEME), benzoylecgonine (BZE), ecgonine methy ester (EME), cocaine (COC), cocaethylene (CE) and nor-cocaine (NC) in BOF.

4.3.4.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ obtained for the detection of EME, AEME, BZE, COC, CE and NC are shown in Table 4.19. The results show that all analytes can be detected up to concentrations as low as 0.1 ng/mL (AEME and CE). LOD ranged from 0.1-0.5 ng/mL for all analytes. The LOQ, on the other hand, was 0.5 ng/mL for all analytes, except for EME that had a LOQ of 1 ng/mL.

Table 4.19 LOD, LOQ and Linearity for the analysis of cocaine and cocaine derivatives in BOF.

Analyte	LOD (ng/mL)	LLOQ (ng/mL)	Calibration range (ng/mL)	Calibration equation	R^2
AEME	0.1	0.5	0.5-250	y=0.783x-0.068	0.998
EME	0.5	1.0	1.0-250	y=0.078x-0.001	0.999
BZE	0.2	0.5	0.5-250	y=0.786x-0.132	0.999
COC	0.03	0.5	0.5-250	y=0.790x+0.045	0.994
CE	0.1	0.5	0.5-250	y=0.775x+0.071	0.992
NC	0.2	0.5	0.5-250	y=0.601x-0.116	0.990

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, NC: Nor-cocaine. LOD: Limit of detection, LLOQ: Low limit of quantification expressed as mean value and %RSD (Percent relative standard deviation) (n = 6).

4.3.4.5 Precision and Accuracy

Within-run precision data for all analytes extracted from BOF are summarised in Table 4.20. The within-run precision values were within the acceptable range of ≤ 20% (SWGTOX 2013) concentration value for the low (0.75 ng/mL), medium (20 ng/mL) and high (200 ng/mL) concentrations.

Table 4.20 Within-run and between-run data for analysis of cocaine and cocaine derivatives in BOF.

		Concentration (ng/mL)								
		AEME			EME			BZE		
		0.75	20	200	0.75	20	200	0.75	20	200
Within-run 1 (n=6)	Mean	0.88	21.0	206	0.88	20.8	233	0.80	17.3	188
	SD	0.04	1.17	16.01	0.04	2.08	39.7	0.00	1.03	17.2
	SE	0.02	0.48	6.53	0.02	0.85	16.2	0.00	0.42	7.04
	RSD	4.62	5.57	7.77	4.62	10.0	15.1	0.00	5.97	9.18
Within-run 2 (n=6)	Mean	0.87	21.2	208	0.82	17.4	195	0.83	17.9	195
	SD	0.08	1.28	10.3	0.26	2.47	22.9	0.10	0.64	10.2
	SE	0.03	0.52	4.21	0.11	1.01	9.34	0.04	0.26	4.18
	RSD	9.42	6.06	4.96	28.8	14.2	11.7	9.99	4.62	5.25
Within-run 3 (n=6)	Mean	0.88	20.7	190	0.92	17.4	195	0.65	16.4	167
	SD	0.04	1.88	16.8	0.26	2.47	22.9	0.05	0.81	14.1
	SE	0.02	0.77	6.87	0.11	1.01	9.34	0.02	0.33	5.75
	RSD	4.62	9.08	8.84	28.8	14.2	11.7	8.43	5.60	8.46
Between-run (n=3)	Mean	0.88	21.0	201	0.87	18.6	208	0.76	17.2	183
	SD	0.01	0.26	9.71	0.05	1.99	21.7	0.10	0.74	14.7
	SE	0.00	0.15	5.61	0.03	1.15	12.5	0.06	0.42	8.50
	RSD	0.66	1.23	4.82	5.76	10.7	10.4	12.7	4.27	8.04
	Accuracy	117	105	101	116	92.8	104	101	86.1	91.6

		COC			CE			NC		
		0.75	20	200	0.75	20	200	0.75	20	200
Within-run 1 (n=6)	Mean	0.82	19.6	185	0.83	19.5	174	0.83	16.9	169
	SD	0.04	1.36	17.8	0.05	1.16	12.7	0.05	0.75	14.7
	SE	0.02	0.56	7.25	0.02	0.47	5.19	0.02	0.30	6.00
	RSD	5.00	7.05	9.59	6.20	5.93	7.29	6.20	5.01	8.67
Within-run 2 (n=6)	Mean	0.88	17.0	160	0.83	17.2	172	0.78	16.6	188
	SD	0.08	1.90	7.79	0.05	1.17	8.87	0.08	0.82	9.30
	SE	0.03	0.78	3.18	0.02	0.48	3.62	0.03	0.34	3.80
	RSD	8.52	11.9	4.84	6.20	6.81	5.82	9.61	6.06	5.89
Within-run 3 (n=6)	Mean	0.82	23.9	196	0.72	22.4	166	0.86	17.5	171
	SD	0.15	2.32	22.6	0.10	1.64	16.8	0.05	1.59	18.1
	SE	0.06	0.95	9.23	0.04	0.67	6.85	0.02	0.65	7.41
	RSD	16.1	8.02	11.55	10.7	6.43	11.5	6.37	9.08	10.6
Between-run (n=3)	Mean	0.84	20.1	181	0.79	19.7	171	0.82	17.0	176
	SD	0.03	3.49	17.9	0.06	2.61	4.59	0.04	0.43	10.3
	SE	0.02	2.02	10.4	0.04	1.51	2.65	0.02	0.25	5.93
	RSD	4.12	17.4	9.93	8.01	13.21	2.69	4.91	2.56	5.83
	Accuracy	112	100	90.3	106	98.7	85.4	109	84.9	88.1

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, CE (Cocaethylene), COC: Cocaine, EME: ecgonine methyl ester and NC (Nor-cocaine). SD; standard deviation, SE: Standard error, %RSD: Percent relative standard deviation.

Precision values were within 15% of the nominal value for all analytes at the three concentrations evaluated except EME that had a %RSD of 29% on two of the three days at the low concentration. The values obtained for AEME were within 9.4%, 9.1% and 8.8% at the low, medium and high concentrations respectively. The values for EME were within 14.2% and 15.1% at the medium and high concentrations. The values for BZE were within 10.0% (Low), 6.0% (Medium) and 9.2% (High). Precision values for COC were within 16.1% (Low), 11.9% (Medium) and 11.6% (High). The values for CE were within 10.7% (Low), 6.8% (Medium) and 11.5% (High). The values for NC were within 9.6% (Low), 9.1% (Medium) and 10.6% (High). Within-run accuracy varied between concentrations and analysis. In general, between-run accuracy was within 4.8% of nominal value for AEME, 10.7% for EME, 12.7% for BZE, 17.4% for COC, 13.2% for CE and 5.8% for NC.

The between-run precision for cocaine in BOF was within the acceptable value of ± 20% (SWGTOX 2013). Precision values were below 4.8, 10.7, 12.7, 17.4, 13.2 and 5.8% for AEME, EME, BZE COC, CE and NC respectively.

4.3.4.6 Carryover

No carryover was detected following the injection of the upper calibrant.

4.3.4.7 Matrix effect

The results of the matrix effect indicated ion suppression or ion enhancement of all analytes from samples in BOF at the three concentrations evaluated. The matrix effect ranged from 78-196 % for all the analytes. The results presented in Table 4.21 showed

ion suppression of EME, COC and CE with matrix effects ranging between 101-196%. Variations (%RSD) in matrix effect between the three concentrations were 16% for EME, 29% for COC and 30% for CE. Ion suppression was also seen at low concentrations of AEME (2%) and NC (22%) in the biological samples. Ion enhancement was observed in AEME and NC at the low and medium concentrations and in BZE at all concentrations evaluated. The ion enhancement ranged between 78-100% with variation (%RSD) of 11% for AEME, 16% for NC and 13% for BZE.

Table 4.21 Matrix effect for the analysis of cocaine and cocaine derivatives in BOF.

Concentration (ng/mL)	Analyte Matrix Effect (%)					
	AEME	**EME**	**BZE**	**COC**	**CE**	**NC**
0.75	102	138	100	174	196	122
20	89	129	78	169	179	89
200	83	101	82	99	105	100
	Ratio of analyte to IS (%)					
	AEME/ AEME-d^3	**EME/ AEME-d^3**	**BZE/ BZE-d^3**	**COC/ COC-d^3**	**CE/ CE-d^3**	**NC/ BZE-d^3**
0.75	103	140	98	90	88	120
20	80	116	101	119	123	112
200	81	98	101	95	96	123

AEME: Anhydroecgonine methyl ester; BZE: Benzoylecgonine; CE: Cocaethylene; COC: Cocaine; EME: ecgonine methyl ester and NC: Nor-cocaine.

Even though ion suppression or enhancement was present in all analytes, the use of deuterated internal standard compensated the matrix effect and therefore the method was not affected by this matrix (Bosker and Huestis 2009). Ratio of analyte to deuterated internal standard (Table 4.21) demonstrated that internal standards were in the same way enhanced or suppressed in the MS source. Mean ratio values over the concentration range were 88, 118, 100, 101, 102 and 118 % for AEME, EME, BZE, COC, CE and NC respectively, resulting in a mean analyte to IS ratio of 104%.

4.3.4.8 Auto-sampler stability

The results of recovery for the auto-sampler stability are presented in Table 4.22. The recovery values for all analytes in BOF were within the acceptable criteria of ± 20% (ranging 96-108%) during a period of four days. The mean recovery values obtained for AEME, EME, BZE, COC, CE and NC in BOF were 102.6 ± 0.9%, 99.8 ± 1.2%, 101.3 ± 0.7%, 100.3 ± 1.4%, 102.0 ± 0.7% and 102.4 ± 1.4% respectively. The results presented in this section confirmed that samples in BOF could be analysed or re-injected within four days following SPE extraction without any significant sample degradation.

Table 4.22 Recovery values for the auto-sampler stability for the analysis of cocaine and cocaine derivatives in BOF.

Day	Concentration (ng/mL)	Recovery (%)					
		AEME	EME	BZE	COC	CE	NC
1	0.75	98.8	96.3	103.0	99.1	102.5	99.6
	20	101.4	99.1	101.3	97.9	100.0	103.6
	200	103.0	97.0	99.8	96.1	100.8	100.2
4	0.75	103.8	100.3	98.9	103.2	100.9	108.4
	20	105.0	102.9	101.4	100.3	105.1	101.4
	200	103.6	103.5	103.4	105.5	102.1	102.1

AEME: Anhydroecgonine methyl ester; BZE: Benzoylecgonine; CE: Cocaethylene; COC: Cocaine; EME: ecgonine methyl ester and NC: Nor-cocaine.

4.4 CONCLUSIONS

Three quantitative LC-MS methods were successfully developed and validated (according to the SWGTOX) for the analysis of cocaine and cocaine derivatives in BOF, porcine oral mucosa and SOF respectively.

The results showed that cocaine and cocaine derivatives could be extracted using the 3 mL TELOS cartridges and the Oasis micro-elution plates with mean recoveries of 98% and 71% respectively. Mean recovery values of 96, 96 and 99% were obtained for AEME, BZE and COC in SOF. The mean recovery values for samples in tissue were 98% for AEME, 99% for BZE and 100% for COC. The mean recovery values for samples in BOF were 56% for AEME, 77% for EME, 57% for BZE, 89% for COC, 100% for CE and 49% for NC.

Identification of analytes was conducted by monitoring a minimum of two target ions for each analyte. Single ion monitoring was used for the detection of analytes (AEME, BZE and COC) in SOF and porcine oral tissue samples. Tandem mass spectrometry was used for detection of analytes (AEME, EME, BZE, COC, CE and NC) in BOF samples. Optimisation of MS parameters allowed the detection of analytes at LOQ of 0.5 ng/mL for AEME and BZE in either SOF or tissue samples. The LOQ obtained for cocaine in SOF or tissue was 1.0 ng/mL. Concentrations of 0.5 ng/mL were obtained as LOQ for AEME, BZE, COC, CE and NC in BOF samples. The LOQ obtained for cocaine in BOF or tissue was 1.0 ng/mL

The results indicated that all methods were robust and sensitive methods for the analysis of cocaine and cocaine derivatives in SOF, oral tissue and BOF. Additionally, these methods allowed high sample throughput, with time limiting steps being the sample preparation, i.e. SPE. The LC-MS methods had run times of eight minutes for SOF and tissue and 5 minutes for BOF. Linear ranges were obtained between 0.5/1.0 – 100 ng/mL for analytes in SOF and tissue and between 0.5-250 ng/mL for BOF, with $R^2 > 0.99$ for all analytes.

The validation results demonstrated that all parameters evaluated, e.g. accuracy, precision, linearity; were within the acceptable values, according with the Scientific Working Group for Forensic Toxicology (SWFTOX) standard practices for method validation in forensic toxicology (SWGTOX 2013). Precision values (between-run and within-run precision) were below the acceptable value of ≤ 20% for all analytes in each matrix. Precision values (%RSD) for AEME, BZE and COC in SOF were below 9.0, 2.8 and 9.1% respectively. The mean values for AEME, BZE and COC in tissue were below 6.7, 9.4 and 10.9% respectively. The mean values for AEME, EME, BZE, COC, CE and NC in BOF were below 9.4, 15.1, 10.0, 17.4, 13.2 and 10.6% respectively. Accuracy values were within ± 20% of nominal value for all analytes in each matrix.

Some carryover was seen after the injection of cocaine samples in SOF and Tissue at high concentrations (>100 ng/mL). This carryover was eliminated by injecting three washes, i.e. blank samples, following the injection of high concentrations of cocaine. No carryover was seen for the rest of analytes in any of the matrices.

Different percentages of matrix effect were obtained for all analytes at different concentrations. All analytes presented either ion suppression or ion enhancement. All analytes presented mean percentages of matrix effect of ± 100%. Mean values for AEME, BZE and COC in SOF were -18, -18 and -59% respectively. Mean values for AEME, BZE and COC in Tissue were -40, -8 and -43% respectively. Mean values for AEME, BZE, COC, CE and NC in BOF were -40, 97, 27, 10 and -40% respectively.

Auto-sampler stability (at room temperature) demonstrated that samples could be analysed up to four days following the extraction of the samples in any of the studied matrices.

Chapter 5

STABILITY OF COCAINE AND COCAINE DERIVATIVES IN BUFFERED ORAL FLUID, SYNTHETIC ORAL FLUID AND PORCINE ORAL MUCOSA

5.1 INTRODUCTION

Knowledge of the Stability of a drug and its metabolites is important to assist in the interpretation of the results obtained following analysis (Kiszka et al. 2000). Skopp (2004, p 91) defined stability as "the capacity of sample material to retain the initial value of a measured quantity for a defined period within specific limits when stored under defined conditions."

Instability of an analyte affects the reliability of the analysis, especially if the samples have been exposed to significant changes in storage temperature or if samples are not being analysed immediately after collection (Drummer 2004). In real toxicology scenarios the stability of the samples needs to be established in order to correlate the results if samples are to be reanalysed (for legal reasons or because of batch failure). Changes in storage conditions can produce the degradation of samples decreasing the concentration of the sample and increase the concentration of their correspondent metabolites (Cone and Menchen 1988).

Studies on the stability of cocaine and metabolites in OF and other matrices such as plasma, blood, phosphate buffer saline and urine have described the degradation of cocaine into benzoylecgonine (BZE) and ecgonine methyl ester (EME), with BZE being the primary degradation product (McCurdy et al. 1989, Hippenstiel and Gerson 1994, Kiszka et al. 2000, 2001, Klingmann et al. 2001).

Few reports on the stability of cocaine (COC) and metabolites in unadulterated or neat oral fluid (OF) have been reported to date (Cone and Menchen 1988, Ventura et al. 2009). Cone and Menchen (1988) reported the stability of cocaine in OF under different

temperature, container and preservatives conditions. The results of this study showed that when cocaine samples were stored in glass or plastic containers and in the fridge, less than 20% of the cocaine degraded after four days of storage. Only when samples were stored (25 °C) in a tube containing sodium citrate samples containing cocaine were stable for seven days. After seven days of storage at 25 °C cocaine degraded in more than 70% (Ventura et al. 2009).

Synthetic OF (SOF) is a good substitute for authentic (neat) OF and it is widely used in research for *in vitro* studies and/or method development (Bosker and Huestis 2009). SOF is often used to overcome the limitation of the low volume and availability when OF is required (Anizan et al. 2015). Although SOF aims to mimic the composition of neat OF, the SOF might contain different constituents or different amounts of constituents than the human OF, including preservatives, stabilising salts and surfactants (Lee and Huestis 2014). These differences in composition between neat OF and SOF could therefore lead to significantly differences in stability of drugs in these matrices (Lee et al. 2012).

The monitoring of stability of cocaine and cocaine derivatives in oral tissue was essential in this research because the concentration of these analytes might change while deposited in the oral cavity and affect the release of drugs in OF. Furthermore, the stability in tissue was evaluated to determine changes in analyte concentration that can be produced from the time of storage, e.g. time passed between sample collection and sample analysis. Stability of cocaine and cocaine derivatives in oral tissues have not been reported to date however, reports have been published relating to cocaine stability in other tissues such as brain, femoral muscle, kidney and liver from deer, rabbits and humans (Moriya and Hashimoto 1996, Kiszka et al. 2001, Rees et al. 2012).

The stability of cocaine and cocaine derivatives reported by these authors showed that cocaine in tissues degraded rapidly when stored at 25 °C and stable when samples were stored in the freezer (-18/-20 °C). Cocaine in human liver, kidney and brain remained stable for 90 days while stored at -20 °C (Kiszka et al. 2001). Samples of homogenised skeletal muscle were stable for 13 months when stored at -18 °C with 15% of the initial concentration decreasing by month 13 (Rees 2011). In samples of liver and kidney stored at 25 °C, the loss in cocaine concentration was significant (35–43%) after one day of storage. After one month of storage at 25 °C, the mean loss was 62% and 47% respectively. The stability of cocaine in human brain at 25 °C after one day was < 10%, after seven days the degradation was about 25% and after one month was about 45%. At 4 °C, the brain tissue was stable for 144 days (Kiszka et al. 2001). Cocaine in skeletal muscle was stable for one month when stored at 25 °C with the NaF (Rees 2011). Cocaine was reported to degrade more rapidly than cocaethylene (CE) in liver tissue (Moriya and Hashimoto 1996).

In this chapter, the influence of temperature (37°C, room temperature, -4°C and 20°C) and time of storage (0-90 days) were examined to determine the stability of cocaine and its metabolites (change in concentration of the analytes) in samples of buffered human OF (BOF), SOF and homogenised porcine oral mucosa. The storage conditions and times presented in this chapter were designed to represent those likely to be encountered during the storage and transportation of biological samples between countries, e.g. Colombia to the UK (Chapter 6) and the conditions under which the *in vitro* studies (Chapter 7) were conducted.

The stability of cocaine and cocaine derivatives in the three matrices was evaluated for as long as three months at different temperatures (room temperature, 4 °C and -20 °C

and at 37 °C). The results of this study were used to check whether BOF samples would be stable during storage and transportation after collection and to aid in the interpretation of results from Chapter 6 and 7.

5.1.1 AIM AND OBJECTIVES

5.1.1.1 Aim:

The aim of this chapter was to investigate the stability of cocaine and cocaine derivatives in BOF, SOF and porcine oral mucosa samples.

5.1.1.2 Objectives:

- To determine the change in concentration of anhydroecgonine methyl ester (AEME), BZE and cocaine in SOF over a period of 3 months at room temperature, 4 °C and -20 °C and at 37 °C (regular body temperature) over a period of four hours.
- To measure the change in concentration of AEME, BZE and cocaine in spiked porcine oral mucosa homogenate at 37 °C over four hours and storage temperature of -20 °C over a period of 30 days.
- To evaluate the best storage conditions for samples containing cocaine and cocaine derivatives (AEME, BZE, EME, cocaethylene CE and nor-cocaine NC) in buffered human oral fluid, i.e. BOF.
- To quantify the degree of degradation of cocaine and metabolites in BOF over a period of two months at room temperature, 4 °C and -20 °C.

5.2 METHODS

5.2.1 Materials

Porcine cheeks were purchased from L F B Meats, Bournemouth, UK.

The analytical standards and reagents used for the analysis of BOF, SOF and tissue were purchased as described in Section 4.2.1.

TELOS® H-CX 130mg/3mL mixed-mode SPE columns were purchased from Kinesis (Cambridgeshire, UK). Oasis mixed-mode cation exchange (MCX) micro-elution plates (Waters, Manchester, UK) were donated by Alere™ Toxicology.

Concateno Certus® oral fluid collection devices were donated by Alere™ Toxicology.

5.2.2 Instrumentation

Analysis of SOF and porcine oral tissue was conducted on an Agilent 1200 Infinity Series LC system coupled to an Agilent Singe Quadrupole 6120 series MS system. Details on the LC-MS systems were described in Section 4.2.2. The analysis was conducted by the researcher at Bournemouth University.

Analysis of BOF samples was carried out using a LC-MS/MS system consisting of a tandem quadrupole mass spectrometer coupled to a Waters Acquity UPLC® system. Details on the LC-MS systems were described in Section 4.2.2. The analysis was conducted by the researcher at Alere Toxicology (Abingdon, UK).

5.2.3 Synthetic Oral Fluid Preparation

SOF was prepared using the Cozart biosciences protocol (2008) "Production of Synthetic Saliva" (Appendix A). SOF was used immediately after preparation or stored at -20 °C until further use.

5.2.4 Homogenised tissue preparation

Preparation of tissue homogenates was described in Section 4.4.1.

5.2.5 Collection of human oral fluid

Samples of control OF were collected from six drug free healthy volunteers (Female and male with age ranging 25-35) using the Concateno Certus® devices (Figure 4.1). The OF was collected following manufacturer recommendations and under the ethical approval granted by Bournemouth University. All collected samples were anonymised by giving a unique code to each sample. After the collection, all devices were stored at room temperature for 24 hours. A pool of human buffered OF (BOF) was subsequently obtained by pooling the collected samples and used to prepare calibration, QCs and stability solutions.

5.2.6 Solution preparation for stability in synthetic oral fluid and porcine oral mucosa

The stability studies were conducted on solutions of SOF or homogenised oral mucosa containing cocaine and cocaine derivatives (AEME and BZE) at a low, middle and high concentration. Solutions of SOF and tissue containing cocaine were prepared individually from AEME and BZE to be able to quantify the degradation of cocaine into BZE. A low

concentration (5 ng/mL) was selected as the 10-fold of the LLOQ of the LC-MS method (Range 0.5 – 100 ng/mL for SOF/Tissue), a medium concentration (20 ng/mL) was selected in the middle of the quantitative range and a high concentration (80 ng/mL) was selected as the 80% of HLOQ of the LC-MS method.

AEME is the pyrolysis product of cocaine and is commonly monitored for the use of crack cocaine (Terms 1990). AEME was included in this chapter because the results from a pilot study developed during this PhD (also presented in Chapter 6) indicated that AEME could be present in OF samples following the consumption/exposure to coca tea.

Initially, the pool of SOF/tissue was divided into six portions (100 mL each). Subsequently, the different portions (portion 1-6) were fortified with the allocated volume of working solution A or B, as indicated in Table 5.1 to obtain final concentrations of 5, 20 and 80 ng/mL analyte in SOF/tissue. The working solution (A) containing cocaine was prepared by diluting 100 µL of drug stock solution (1 mg/mL) in 1 mL methanol to obtain a concentration of 0.1 mg/mL. The second working solution (B) containing a mixture of AEME and BZE was prepared by diluting 100 µL of each drug stock solution (1 mg/mL) in 1 mL methanol to obtain a concentration of 0.1 mg/mL.

Table 5.1 Preparation of SOF and Tissue stability samples.

Concentration of analytes in SOF/Tissue (ng/mL)		SOF/Tissue Portion	Working solution	Volume Working solution (µL)
LOW	5	1	A	5
		2	B	5
MIDDLE	20	3	A	20
		4	B	20
HIGH	80	5	A	80
		6	B	80

SOF: Artificial oral Fluid, A: Solution containing cocaine (COC), B: Solution containing anhydroecgonine methyl ester (AEME) and benzoylecgonine (BZE), ng/mL: nano-gram per millilitre, µL: microliter, Tissue: porcine oral tissue.

Twenty aliquots (3.5 mL each) containing cocaine or the mixture of drugs were obtained for each concentration level. The volume of the aliquots allowed the analysis of analytes in triplicate. All aliquots (20 aliquots x 3 concentrations = 60 aliquots in total) were stored in 60 individual 5 mL glass tubes.

5.2.7 Solution preparation for stability in buffered oral fluid

The stability studies were conducted on solutions of BOF containing cocaine or cocaine derivatives AEME, BZE, EME, CE and NC at a low, middle and high concentration. Solutions of BOF containing cocaine were prepared individually from cocaine derivatives to be able to quantify the degradation of cocaine into BZE and EME. The low concentration (5 ng/mL) was selected as the 10-fold of the LLOQ of the LC-MS method (Range 0.5-100 ng/mL for BOF), a medium concentration (50 ng/mL) was selected in the middle of the quantitative range and a high concentration (200 ng/mL) was selected as the 80% of HLOQ of the LC-MS method.

Initially, the pool of BOF was divided into six portions (25 mL each). Subsequently, the different portions (portion 1-6) were fortified with the allocated volume of working solution A or B, as indicated in Table 5.2 to obtain final concentrations of 5 ng/mL, 50 ng/mL and 200 ng/mL analyte in BOF. The working solution (A) containing cocaine was prepared in methanol to obtain a concentration of 10 μg/mL from a 100 μg /mL stock solution. The second working solution (B) containing a mixture of AEME, BZE, EME, CE and NC, was prepared in methanol to obtain a final concentration of 10 μg/mL for each analyte from a 100 μg /mL independent stock solutions.

Table 5.2 Preparation of BOF and porcine oral tissue stability samples.

Concentration of analytes in BOF (ng/mL)		BOF Portion	Working solution	Volume working solution (μL)
LOW	5	1	A	15
		2	B	15
MIDDLE	50	3	A	150
		4	B	150
HIGH	200	5	A	600
		6	B	600

BOF: Buffered Oral Fluid, A: Solution containing cocaine (COC), B: Solution containing anhydroecgonine methyl ester (AEME), benzoylecgonine (BZE), ecgonine methyl ester (EME), cocaethylene (CE) and nor-cocaine (NC), ng/mL,: nano-gram per millilitre, μL: microliter.

Twenty aliquots (1.3 mL each) containing cocaine or the mixture of drugs were obtained for each concentration level. The volume of the aliquots allowed the analysis of analytes in triplicate. All aliquots (20 aliquots x 3 concentrations = 60 aliquots in total) were stored in 60 individual 5 mL plastic tubes.

5.2.8 Storage conditions and time of storage

Stability samples prepared in BOF, SOF and homogenised porcine oral mucosa were prepared at different conditions. The time of storage varied depending on the conditions under which the *in vivo* (Chapter 6) and *in vitro* (Chapter 7) studies were developed:

Samples prepared in SOF were divided into four groups and stored at four different temperatures: 37 °C, room temperature (18-20 °C), 4 °C and -20 °C, as shown in Figure 5.1. The times selected for the analysis of SOF at room temperature, 4 °C and -20 °C were 0, 1, 4, 7, 14, 30, 60 and 90 days. The times selected for the analysis at 37 °C were 0, 0.25, 0.5, 1, 2, 4 and 24 hours. Samples stored at 37 °C were analysed over a period of 24 hours because the *in vitro* diffusion studies (Chapter 7) were conducted over this period of time.

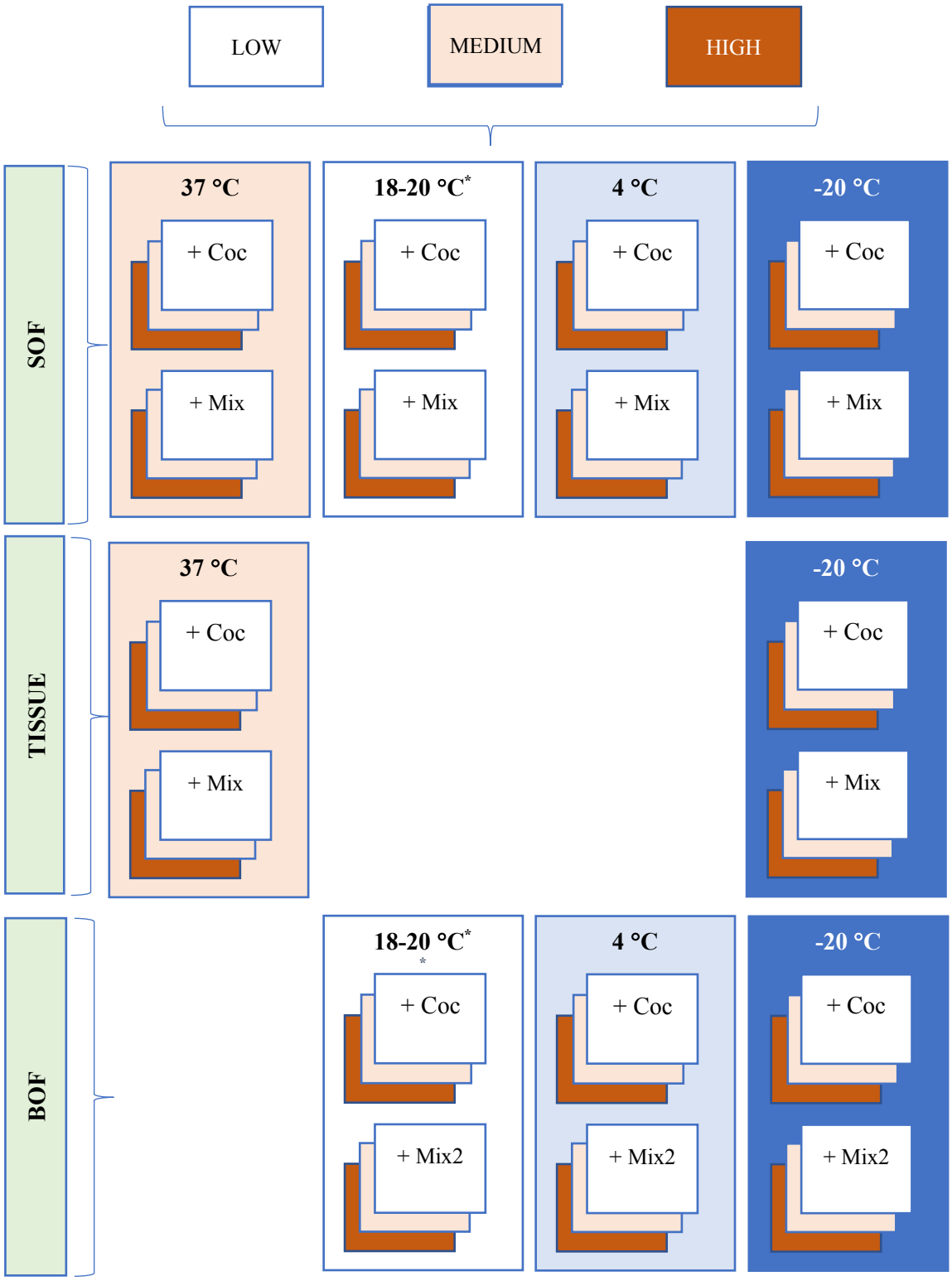


Figure 5.1 Diagram of preparation and storage of SOF, porcine oral tissue and BOF samples containing cocaine and cocaine derivatives. (+Coc) Addition of COC. (+ Mix) Addition of AEME and BZE. (+ Mix2) Addition of AEME, BZE, CE and NC. (*n*) Number of samples. *Stability on day 11 was only evaluated in samples stored at room temperature.

Stability samples prepared in homogenised tissue were divided into two groups and stored at two different temperatures: 37 °C and -20 °C (Figure 5.1). The times selected for the analysis of tissue at 37 °C were 0, 0.25, 0.5, 1, 2, 4 and 24 hours. The times for the analysis of samples stored at -20 °C were 0, 1, 4, 7, 14 and 30 days. Samples stored at 37 °C were analysed over a maximum period of 24 hours because the *in vitro* diffusion studies (Chapter 7) were conducted over this period of time. Samples stored at -20 °C were analysed for a longer time (30 days) to allow enough time for the analysis. Tissue samples were stored for a maximum period of 30 days before being analysed by LC-MS.

Stability samples prepared in BOF were divided into three groups and stored at three different temperatures: room temperature, 4 °C and -20 °C (Figure 5.1). The times selected for the analysis were 0, 1, 4, 7, 14, 30 and 60 days. These conditions were designed to represent those likely to encounter during transportation between Colombia and the UK and to evaluate the storage of samples for a maximum period of two months.

5.2.9 Sample analysis

Samples prepared in SOF and homogenised porcine tissue were extracted and analysed using the validated methods described in Chapter 4 (Section 4.3.4). All samples were analysed using calibrators and QCs prepared on the day of analysis. The analysis was conducted in triplicate on the selected days (Section 5.2.8). The samples were thawed at room temperature and extracted within the day and left to run on the LC-MS instrument overnight.

Samples in BOF were extracted and analysed using the validated method described in Chapter 4 (Section 4.3.4). All samples were analysed using calibrators and QCs prepared

on the day of analysis. The analysis was conducted in triplicate on the selected days (Section 5.2.8). The samples were thaw at room temperature and extracted within the day and left to run on the LC-MS/MS instrument overnight.

5.2.10 Evaluation of stability of analytes

Analytes analysed on day 0 were held as baseline or 100% concentrations. Changes in concentration were presented as percentage of day 0 (% baseline) and were determined as: (mean stored sample concentration) / (mean baseline concentration) x 100, with n = 3. Concentration changes < 20% were considered stable (Lee et.al. 2013).

Stability of cocaine was assessed over time by quantifying the change in concentration of the parent compound and its major metabolite, BZE.

5.2.11 Data analysis

Non-parametric Spearman's test was used for correlation analysis (IBM SPSS Statistics version 23). Results with 2-tailed $p_s < 0.05$ were considered significant.

5.3 RESULTS AND DISCUSSION

5.3.1 Stability of cocaine, AEME and BZE in synthetic oral fluid

Results of the stability of AEME and BZE in SOF are illustrated in Figure 5.2. These results showed that AEME and BZE were stable at -20 ºC (Figure 5.2 a-c) for up to 90 days at the low, medium and high concentrations in SOF. All concentrations were within 20% from the original AEME and BZE concentrations, with mean values of 92.6% (82.3-

103.4%) for AEME and 90.2% (79.5-95.4%) for BZE. Concentration variability (%RSD) of AEME and BZE was < 8.2%. Changes in concentration of less than ± 20% of the baseline (Day 0) accounted for analytical imprecision (Lee et al. 2012). Variations in the pipetted volumes of samples could have also contributed to the variations in concentration. When samples are pipetted at different temperatures, e.g. the liquid temperature or the ambient temperature, the volume of the sample dispended varies (an increase of temperature might lead to a reduction in the volume dispensed) (Ewald 2015). Even though all samples were thawed at room temperature for over one hour, the ambient temperature could have varied across the study and therefor the temperature and transferred volume of the SOF samples. SOF as well as homogenised tissue and BOF contained more than 90% water, which density (mass/volume) decreases with the increase in temperature.

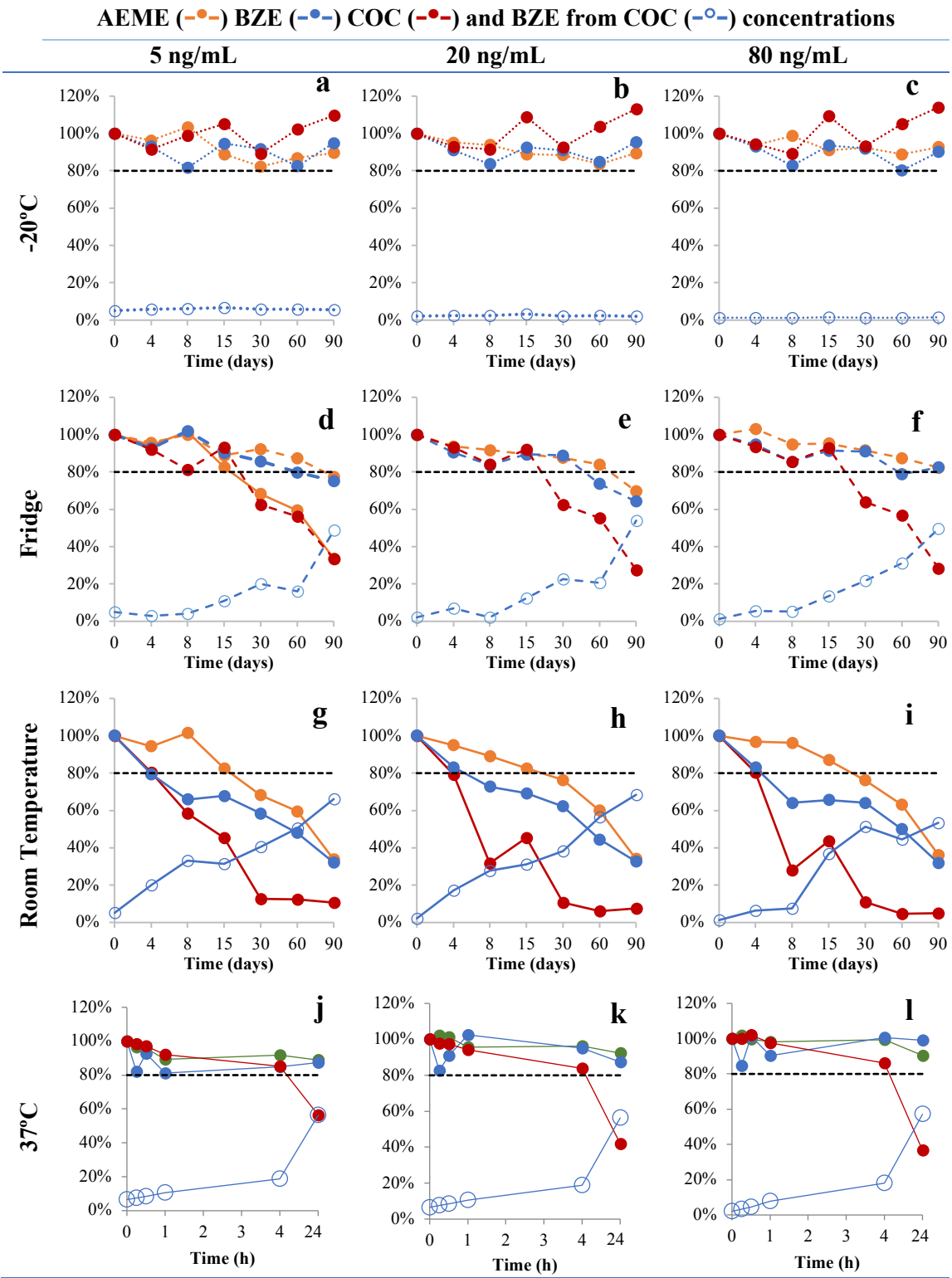


Figure 5.2 Stability of AEME, BZE and cocaine in SOF. Concentration as percentage of day 0. Stability at -20°C for the concentrations 5 ng/mL (a), 20 ng/mL (b) and 80 ng/mL (c). Stability in the fridge for the concentrations 5 ng/mL (d), 20 ng/mL (e) and 80 ng/mL (f). Stability at room temperature for the concentrations 5 ng/mL (g), 20 ng/mL (h) and 80 ng/mL (i). Stability at 37°C for the concentrations 5 ng/mL (j), 20 ng/mL (k) and 80 ng/mL (l). Dotted lines at 80% indicate the stability limit of 20% variation from day 0.

Storage at 4 ºC (Fridge stability) indicated that AEME was stable for up to 60 days at the low and middle concentrations (Figure 5.2 d-e) and stable up to 90 days for the high concentration (Figure 5.2 f). A decrease in AEME mean concentration of 22.5% and 30.4% were seen for the low and middle concentration on day 90. The decrease in AEME concentration could have been the result of degradation into anhydroecgonine (AE) during storage (Fandiño et al. 2002). BZE was stable for up to 30 days at all concentrations. Samples of BZE at the high concentration were stable on day 90 (mean relative concentration of 82.5%). A decrease in BZE concentration of 22.6% (20.2-26.4%) and 26.0% (17.5-35.8%) was seen on day 60 and 90 respectively (%RSD: 14.7 and 35.6% respectively).

Stability at 18-20 ºC (room temperature) indicated that AEME was stable for up to 15 days at all of the three concentrations evaluated, with values within ± 20% of day 0. The recovery values were 82.6%, 82.6 and 87.0% for the low, medium and high concentrations on day 15. The %RSD within the 15 days was < 7.3 for all concentrations. A decrease in mean concentration of 26.4% (23.7-31.8%), 39.2% (36.9-40.5%) and 65.4% (66.0-63.8%) was seen on day 30, 60 and 90 respectively, indicating its degradation into AE. BZE was stable up to four days at the middle and high concentrations (recovery values of 83.1% and 83.0% respectively). Stability at the low concentration (79.4%) indicated that BZE in SOF was stable only for few hours. Analytical errors could have produced the small changes in concentration in relation to Day 0 (20.6%) indicating that BZE samples were stable up to four days. Stability of BZE and AEME could not be obtained on day 1, because of instrument failure, therefore it was not possible to confirm that BZE (5 ng/mL) was stable for 24 hours. A decrease in mean concentration of 67.8% (67.4-68.1%) was seen for BZE after 90 days of storage at room temperature.

Stability at 37 ºC (Figure 5.2 j-l) indicated that AEME and BZE were stable for up to 24 hours in SOF at all concentrations evaluated, with changes below 20% from day 0. All concentrations were within 20% of the original AEME and BZE concentrations, with mean values of 96.7% (88.9-102.2%) for AEME and 91.7% (81.1-101.3%) for BZE. Concentration variability (%RSD) of AEME and BZE was 4.5% and 7.1% respectively.

The results indicated that the concentration of BZE decreased as the temperature of storage increased after few days of storage. This decrease in concentration corresponded to the degradation of BZE in the fridge and at room temperature. Chen et al. (2016) reported ecgonine (ECG) as the hydrolysis product of BZE following enzymatic degradation. ECG was not analysed in this study; therefore, it could not be confirmed as the degradation product of BZE.

BZE is the hydrolysis product of cocaine and concentrations of BZE in SOF samples were related to the degree of hydrolysis of cocaine (Klingmann et al. 2001, Cognard et al. 2006). The results presented in Figure 5.2 confirmed this statement by showing that there was a decrease in cocaine concentration compensated by the appearance of BZE in SOF samples over a period of 90 days at different storage conditions.

Samples of cocaine in SOF stored in the freezer (-20 ºC) were stable for the length of the study (90 days) at the three concentrations (Figure 5.2 a-c), with a mean recovery value of 100.2% (89.1-114.1%). The variability (%RSD) of cocaine concentration was 7.3%, 8.5% and 9.1% for the low, middle and high concentrations respectively. The variations in cocaine concentration were attributed to analytical imprecision and random errors from the volume of the samples analysed as it was previously described (Lee et al. 2012, Ewald 2015). BZE could not be quantified as it was detected at concentrations

below the LOQ of the method. These results indicated that cocaine did not degrade when samples of SOF were stored at -20 ºC.

When samples were stored in the fridge (-4 ºC) (Figure 5.2 d-f), the stability of cocaine decreased, and samples were stable up to 15 days (92.7%). A mean decrease in concentration of 37.1% (36.2-37.5%), 43.9% (43.2-44.6%) and 70.2% (66.5-72.4%) was seen in day 30, 60 and 90 respectively. As can be observed in Figure 5.2 (d-f) when the concentration of cocaine decreased, the concentration of BZE increased consequently. The concentration of BZE increased up to 54% of the initial cocaine concentration over 90 days. The percentage of BZE in relation to the cocaine concentration (day 0) ranged 2.9-48.7%, 2.1-53.9% and 1.3-49.6% for the low, medium and high concentrations respectively. The decrease in concentration of cocaine was significantly correlated with the increase in concentration of BZE when samples were stored at room temperature at the medium and high concentrations (Medium: r_s = -0.86, p = 0.01; High: r_s = -0.89, p = 0.007), whereas no significant correlation was obtained for the samples at the low concentration (Low: r_s = -0.64, p = 0.119). These results indicated that at highest temperatures the degradation of cocaine into BZE is more significant that at low concentrations.

Room temperature experiments (Figure 5.2 g-i) demonstrated that cocaine in SOF was stable up to four days at the low and high concentrations (80.3% and 80.3% respectively). The medium concentration appeared to be instable after four hours of storage (79.2%) suggesting that cocaine in SOF was stable for only few hours. Analytical errors could have produced the small changes in concentration in relation to Day 0 (20.8%) indicating that cocaine samples were stable for up to four days. The stability at day 1 could not be obtained because of instrument failure, therefore, it was not possible to confirm that

cocaine was stable for al least 24 hours of storage at the medium concentration. On day 90, a degradation of > 90% was observed for cocaine, with concentrations of cocaine decreasing by 89.4%, 92.4% and 95.1% for the low, medium and high concentrations respectively. On the contrary, an increase in the concentration of up to 68.4% was seen for BZE on this day 90. Mean concentrations (as percentage of cocaine day 0) ranged from 5.1-66.1%, 2.1-68.4% and 1.3-53.4% for the low, medium and high concentrations respectively. The concentration of cocaine was significantly correlated with the concentration of BZE when samples were stored at room temperature (Low: r_s = -0.96, p < 0.001; Medium: r_s = -0.93, p = 0.003; High: r_s = -0.86, p = 0.01).

The stability at 37ºC (Figure 5.2 j-l) indicated that cocaine in SOF was stable up to four hours at the low, medium and high concentrations (94.5%, 94.7% and 97.1% respectively). Mean concentrations ranged from 85.1-100.0%, 83.9-100.0% and 86.0-100.0% for the low, middle and high concentrations respectively. BZE was detected as break down product of COCAINE (Klingmann et al. 2001, Cognard et al. 2006) at concentrations up to 18.8% (Low), 17.1% (Middle) and 17.9% (High) (as percentage of COCAINE day 0). After 24 hours at 37 ºC, the concentration of cocaine decreased in more than > 64%, with concentrations of cocaine decreasing by 43.5%, 58.0% and 63.3% for the low, medium and high concentrations respectively. The concentration of BZE consequently increased up to 57% after 24 hours storage at 37ºC. Mean concentrations of BZE in SOF (as percentage of cocaine day 0) ranged from 6.5-56.3%, 2.8-56.8% and 2.1-57.1% for the low, medium and high concentrations respectively.

Overall, the increase in BZE concentration did not correspond to the concentration of cocaine decreasing. Whereas cocaine concentration decreased up to 95% on day 90, BZE increased up to 68%. These discrepancies could be attributed to the additional degradation

of cocaine into EME and subsequently into ECG (Klingmann et al. 2001). Since EME and ECG were not included in the quantitative method used to evaluate the stability of analytes in SOF, this result could not be confirmed.

5.3.2 Stability of cocaine, AEME and BZE in porcine oral mucosa

In this chapter, the stability of cocaine and metabolites was evaluated in tissue in order to aid the interpretation of results from *in vitro* diffusion studies (Chapter 7). Even though the diffusion studies were conducted in pieces of whole tissue, for accuracy purposes, the stability of cocaine and cocaine derivatives were conducted in homogenised tissue. This procedure was preferred as spiking of individual pieces of tissue resulted in different absorption of cocaine and therefore variances in the amount of cocaine present in different sections of the tissue (RSD >30%). The degradation of cocaine in biological tissue has been reported for its application in post-mortem analysis (Moriya and Hashimoto 1996, Kiszka et al. 2001, Rees et al. 2012).

Stability studies were conducted at 37 ºC and -20 ºC storage temperature only. These conditions were selected as the *in vitro* studies were conducted at 37ºC for a period of time of up to four hours and samples were stored at -20 ºC until quantitative analysis. Stability at room temperature and -4 ºC was not evaluated because there were not experiments conducted at these temperatures or tissue samples stored under these conditions. The results of the stability of AEME, BZE and cocaine at 37 ºC and -20 ºC are shown in Figure 5.3.

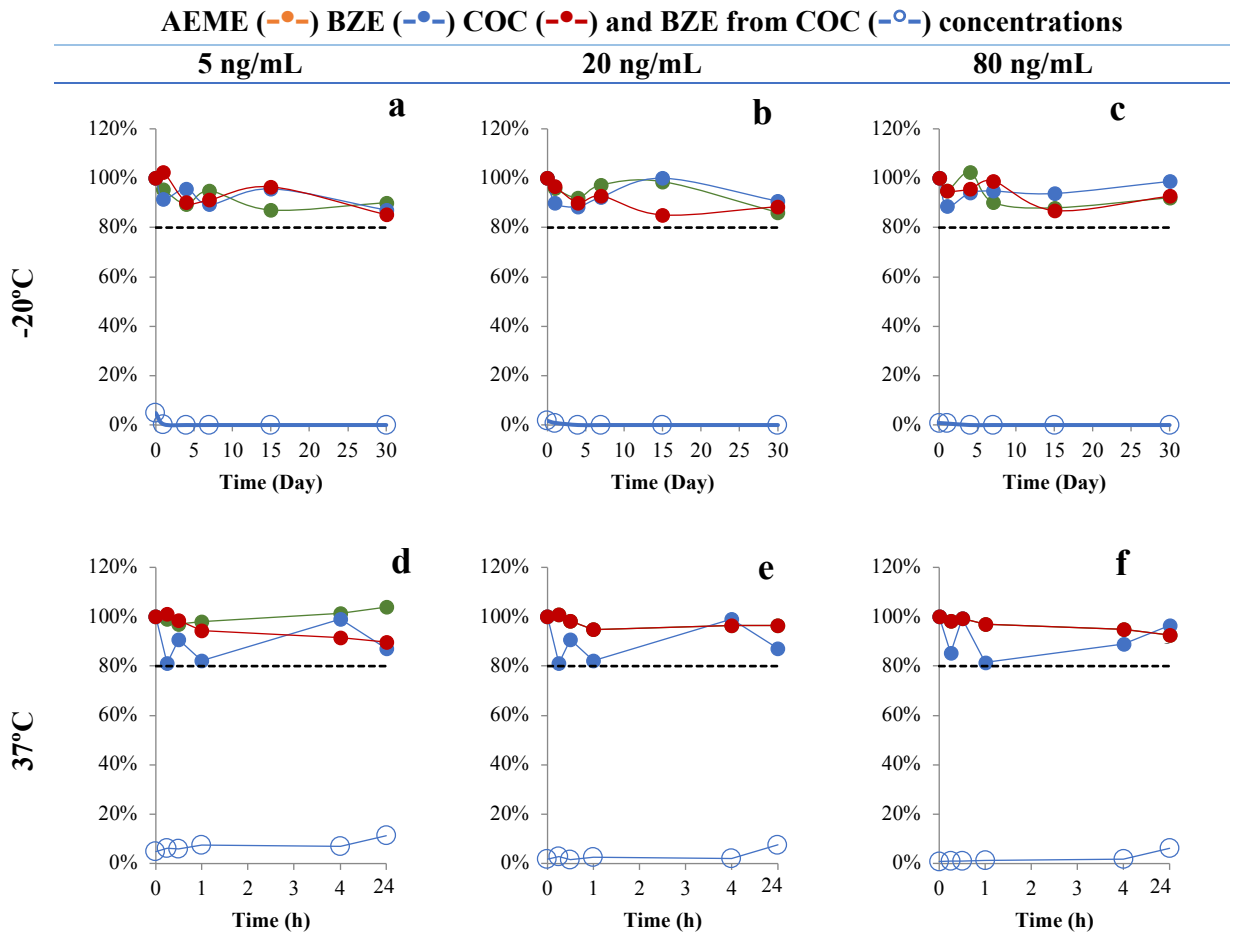


Figure 5.3 Stability of AEME, BZE and cocaine in homogenised porcine oral mucosa. Concentration as percentage of day 0. Stability at -20°C for the concentrations 5 ng/mL (a), 20 ng/mL (b) and 80 ng/mL (c). Stability at 37°C for the concentrations 5 ng/mL (d), 20 ng/mL (e) and 80 ng/mL (f). Dotted lines at 80% indicate the stability limit of 20% variation from day 0.

Stability showed that AEME and BZE analytes were stable at -20 ºC for up to 30 days at the low, medium and high concentrations in homogenised tissue (Figure 5.3 a-c). All concentrations were within ± 20% from the original AEME and BZE concentrations, with mean relative recovery values of 94.2% (86.1-102.5%) for AEME and 94.0% (87.1-100.0%) for BZE. Concentration variability (RSD%) for AEME and BZE were 5.3% and 4.4%. This variability could have been the results of analytical imprecision (Lee et al. 2012) and variations in the pipetted volumes of the samples from the fluctuations in the temperature of the samples (Ewald 2015).

The Stability at 37 °C (Figure 5.3 d-f) demonstrated that samples of porcine oral tissue containing AEME and BZE were stable for four hours at the low, medium and high concentrations. All concentrations were within ± 20% from the original AEME and BZE concentrations, with mean relative recovery values of 98.3% (88.9-102.2%) for AEME and 102.5% (81.1-101.3%) for BZE. The recovery values of AEME were 99.9% (97.0-103.9%) for the low concentration, 97.5% (93.2-101.8%) for the medium concentration and 97.5% (95.4-98.8%) for the high concentration. The recovery values for BZE were 90.0% (81.1-99.0%), 93.1% (82.7-102.5%) and 92.0% (81.5-99.4%) for the low. Medium and high concentrations respectively. Concentration variability (%RSD) of AEME and BZE recovery values were 2.8% and 7.3% respectively.

Samples of cocaine at the three concentrations in homogenised tissue (Figure 5.3 a-c) were stable for the length of the study (30 days) when stored in the freezer (-20 ºC). Mean values of 96.1%, 92.2% and 94.8% for the low middle and high concentrations were obtained respectively (giving an overall mean of 98%). The variability (%RSD) of cocaine concentration was 6.8, 5.9 and 5.0% for the low, middle and high concentrations respectively. The concentration of BZE as a percentage of cocaine day 0 was below 5% and ranged from 0.3-4.8%. These results indicated that no significant degradation of cocaine in this study was obtained when samples were stored at -20 ºC.

The stability at 37 ºC (Figure 5.3 d-f) indicated that cocaine in porcine oral tissue was stable for the length of the study (four hours) for the low, medium and high concentrations (97.0, 98.1 and 97.9% respectively). The variability (%RSD) of cocaine concentration was 4.8%, 2.4% and 2.9% for the low, middle and high concentrations respectively. Additionally, an increase in the concentration of up to 6.8% was seen for BZE after four

hours. Mean concentrations (as percentage of cocaine day 0) ranged from 4.8-6.8%, 1.7-2.0% and 0.8-1.8% for the low, medium and high concentrations respectively.

In contrast with the SOF analysis, the amount of BZE being formed, i.e. appearing in the tissue samples containing cocaine agreed to the loss of cocaine concentration, with %RSD values < 3%. Hence, indicating that the quantification of BZE could account small losses of cocaine.

5.3.3 Stability of cocaine and cocaine derivatives in buffered oral fluid

The stability of cocaine derivatives (AEME, BZE, CE, EME and NC) in BOF is illustrated in Figure 5.4. Stability showed that AEME, CE and NC analytes were stable at -20 ºC (-24.9 ± 0.6 °C) for up to 60 days at the low, medium and high concentrations in BOF (Figure 5.4 a-c). Excepting the mean concentration of NC at the medium concentration on day 15 that was 23.6% from that of day 0. Concentration variability (RSD%) for all analytes were < 17.7%. These variations could have been the result of analytical imprecision (Lee et al. 2012) and variations in the pipetted volumes of the samples from the fluctuations in the temperature of the samples (Ewald 2015) as discussed in Section 5.3.1.

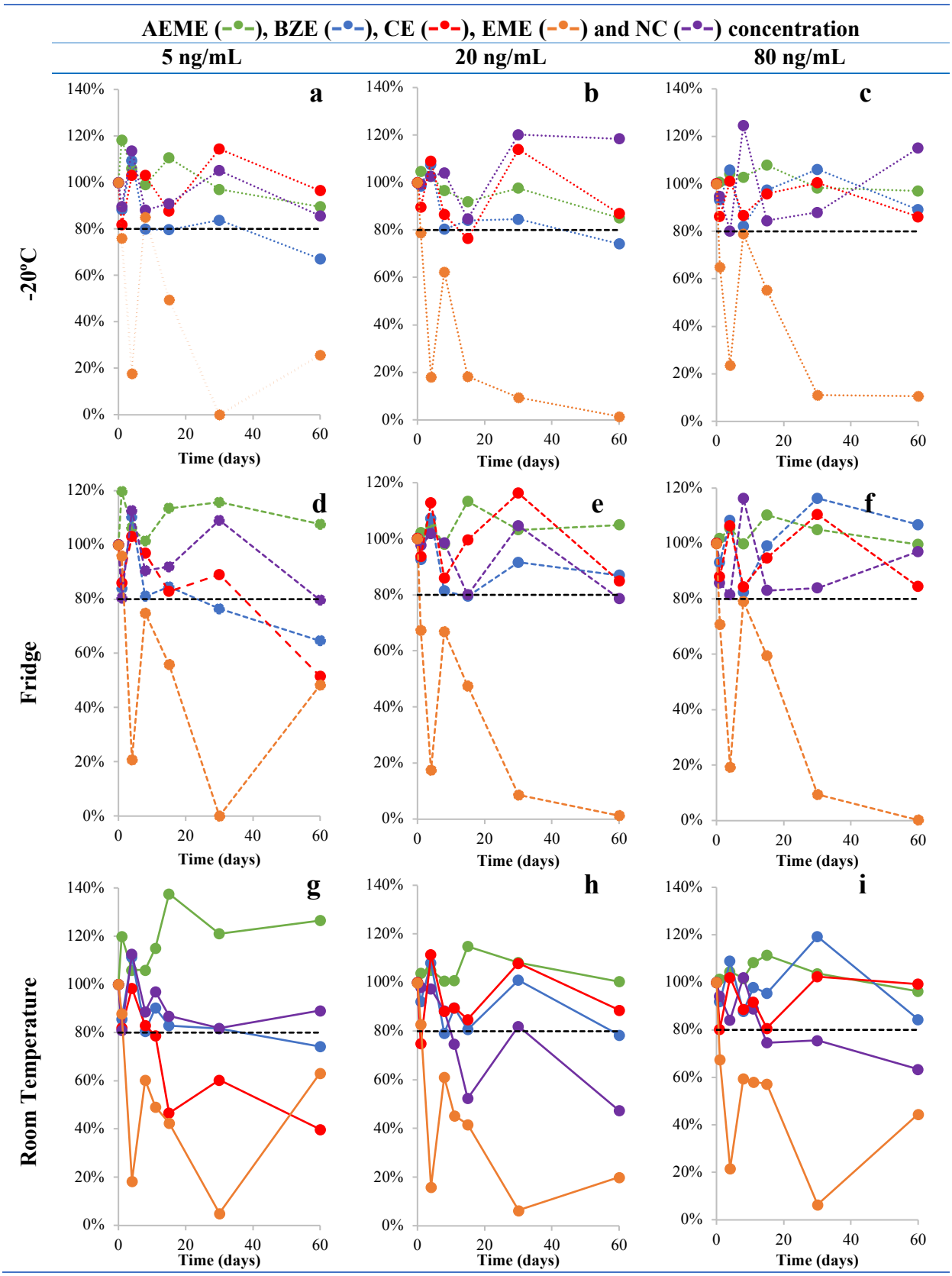


Figure 5.4 Stability of AEME, BZE, EME, CE and NC in BOF. Concentration as percentage of day 0. Stability at -20°C for the concentrations 5 ng/mL (a), 50 ng/mL (b) and 200 ng/mL (c). Stability in the fridge for the concentrations 5 ng/mL (d), 50 ng/mL (e) and 200 ng/mL (f). Stability at room temperature for the concentrations 5 ng/mL (g), 50 ng/mL (h) and 200 ng/mL (i). Dotted lines at 80% indicate the stability limit of 20% variation from day 0.

Solutions of BZE at low and medium concentration were stable up to 30 days at -20 °C, and solutions at a high concentration were stable up to 60 days. The stability of EME indicated that this compound was only stable for few hours (less than 24 hours) at any of the three concentrations. A decrease of 74.4%, 98.5% and 89.4% was seen for the low, medium and high concentration respectively on day 60. These results could be the result of the breakdown of EME into ECG (Klingmann et al. 2001).

Samples stored in the fridge (5.2 ± 0.4 °C) (Figure 5.4 d-f) were stable for up to 60 days for AEME at all concentrations. Concentrations of AEME in BOF presented a percentage of recovery of 109.2% (101.4-119.7%) (low), 103.7% (98.1-113.3%) (Medium) and 103.2% (99.6-105.6%) (High), over the 60 days. Concentration variability (RSD%) was < 2.6%. Samples of BZE at the medium (91.3%, 81.4-107.3%) and high (100.9 %, 93.2-116.3%) concentrations were stable up to 60 days. However, samples at the low concentration appeared to be stable only up to 15 days (92.0 %, 81.1-110.3%). A decrease of BZE concentration of 23.6% and 35.4% was seen for the low concentration on days 30 and 60 respectively. Solutions containing CE were stable up to 30 days for the low (93.0%, 82.9-103.1%) and medium (97.1%, 80.0-101.8%) concentrations and stable up to 60 days for the high concentration (92.5%, 81.5-116.3%). Similarly, to the stability at -20 ºC, EME was stable for less than 24 hours at the medium and high concentration and stable up to one day at the low concentration. A decrease of up to 99% was seen for all concentrations at the time of the study. NC was stable for up to 60 days at all concentrations, with mean concentrations of 94.9% (79.6-112.7%) (Low), 99.0% (84.9-112.8%) (Medium) and 95.5% (84.4- 110.5%).

Stability at room temperature (Figure 5.4 g-i) indicated that BOF containing AEME were stable for up to 11 days at the low concentration (109.4%, 105.9-119.9%) and stable

up to 60 days at the medium (104.3%, 100.0-114.9%) and high (103.4%, 96.2-111.4%) concentrations. The variation in concentration of AEME on day 15, 30 and 60 ranged 121-138% from day 0. These high percentages were attributed to the instrument performance, i.e. ion enhancement in the MS detection, and no to degradation of the sample or experimental error (the transference (pipetting) of the sample, SPE process or reconstitution volume). This statement can be supported by the fact that the other analytes present in the same sample did not show high percentages of variation. Ion enhancement is a common disadvantage of ESI and is caused by the competition between ions to be expelled from the droplet during the desolvation process (Particle sciences 2009).

Concentrations of BZE at the low and medium values, on the other hand, were stable up to 30 days with percentages of 90.3% (80.5-111.3%) and 93.0% (80.8-108.0%) respectively. Excepting day eight (medium concentration) that appeared to be unstable, with a loss of concentration of 20.7%. At the high concentration, BZE was stable up to 60 days (98.2%, 84.4-119.2%). The stability of CE was eight days for the low and medium concentrations and 11 days for the high concentration. Percentages of variation were 90.8% (82.9-98.4%), 96.0% (88.4-98.0%) and 93.9% (84.1-102.1%) respectively. A decrease in mean concentration of up to 60.1%, 52.6% and 36.6% were seen in the low, medium and high concentrations on day 60. NC was stable for up to 60 days at all concentrations (Low: 92.1%, 80.9-112.6 %; Medium: 93.2%, 84.8-111.4% and High: 93.1%, 80.3-102.4%).

The stability of cocaine in BOF is presented in Figure 5.5. This figure illustrates the change in concentration of cocaine and BZE which is the main break down product of cocaine (Cognard et al. 2006). Samples of cocaine in BOF stored in the freezer (-20 ºC) (Figure 5.5 a-c) were stable for the length of the study (60 days) at the three

concentrations. Excepting cocaine at the medium concentration on day 30, where the percentage of recovery was 126.9%. This high value could have been the result of experimental error: (1) pipetting error: a higher volume being analysed could have led to the quantification of higher amounts of analyte (Ewald 2015). (2) The volume of the solution used in the reconstitution step, which could have affected the final concentration (Ewald 2015). (3) Ion enhancement from the MS source (Particle sciences 2009).

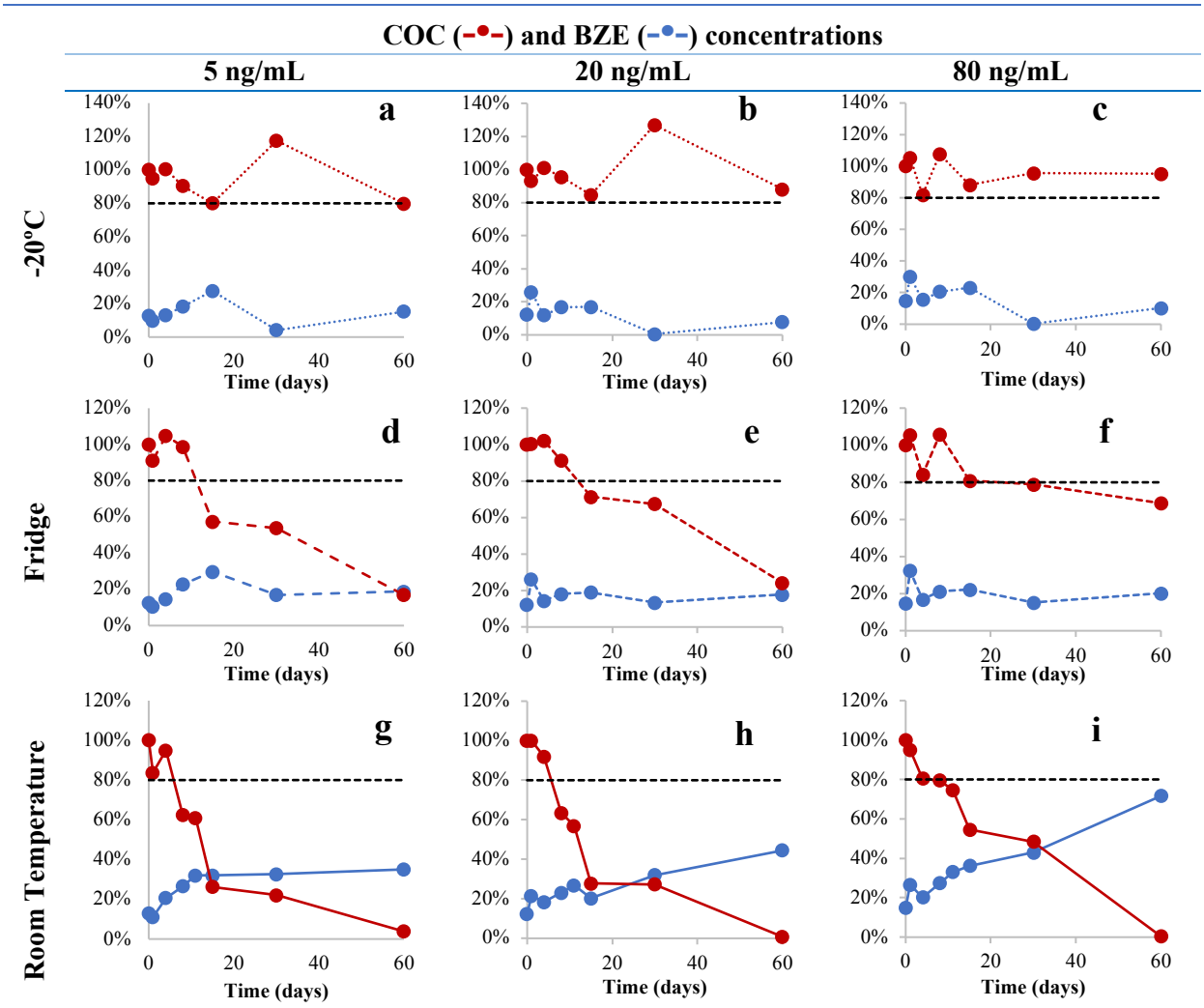


Figure 5.5 Stability of cocaine and BZE in BOF. Concentration as percentage of cocaine day 0. Stability at -20°C for the concentrations 5 ng/mL (a), 50 ng/mL (b) and 200 ng/mL (c). Stability in the fridge for the concentrations 5 ng/mL (d), 50 ng/mL (e) and 200 ng/mL (f). Stability at room temperature for the concentrations 5 ng/mL (g), 50 ng/mL (h) and 200 ng/mL (i). Dotted lines at 80% indicate the stability limit of 20% variation from day 0.

The mean concentration values of cocaine were 94.7% (79.8-117.5%), 98.4% (84.6-126.9%) and 96.2% (81.8-107.6%) for the low, middle and high concentrations respectively. The mean concentration of BZE as a percentage of cocaine on day 0 was < 20% and ranged from 13.2-16.4% within the three concentrations. These results indicated that cocaine was not significantly hydrolysed into BZE when samples of SOF were stored at -20 ºC. The concentration of BZE was not significantly correlated with the concentration of cocaine at any of the concentrations (Low: r_s = -0.75, p > 0.05; Medium: r_s = -0.57, p > 0.05 and High: r_s = 0 .21, p > 0.05), therefore confirming the low degradation of cocaine in the BOF samples.

When samples were stored in the fridge (Figure 5.5 d-f), the stability of cocaine decreased, and samples were stable up to 15 days at the low and medium concentrations and up to 30 days at the high concentration. A mean decrease in concentration of 83.1%, 75.8% and 31.3% were seen on day 60 at the low, medium and high concentrations respectively. Even though the concentration of cocaine decreased over time, the concentration of BZE did not increase notably (Figure 5.5). The BZE concentration increased up to 32% of the initial cocaine concentration over the 60 days. The percentage of BZE in relation to the cocaine concentration (day 0) ranged 7.7-29.8%, 12.4-26.1% and 14.8-32.3% for the low, medium and high concentrations respectively. No correlation between the decrease in the concentration of cocaine with the increase in concentration of BZE was seen at any of the three concentrations (Low: r_s = -0.39, p > .05; Medium: r_s = -0.07, p > 0.05; High: r_s = -0.29, p > 0.05). These results indicated that the increase of BZE was not representative of the decrease in cocaine concentration.

The stability at room temperature demonstrated that solutions of cocaine in BOF were stable up to four days at low (92.9%), medium (97.3%) and high (91.8%) concentrations.

On day 60, a cocaine degradation of > 90% was seen, with concentrations of cocaine decreasing up to 96.2%, 99.2% and 99.6% at the low, medium and high concentrations respectively. On the contrary, an increase in the concentration of up to 72% was seen for BZE on this day (Day 60). Mean concentrations (as percentage of cocaine day 0) ranged from 11.1-35.0%, 12.4-44.5% and 14.8-71.6% for the low, medium and high concentrations respectively. The decrease in concentration of cocaine was significantly correlated with the increase in BZE concentration when samples were stored at room temperature (Low: r_s = -0.92, $p < 0.001$; Medium: r_s = -0.74, $p < 0.05$; High: r_s = -0.98, $p < 0.001$).

A decrease of up to 99% in cocaine was accompanied by an increase in BZE concentration of up to 72% on day 90, indicating that the amount of BZE formed in the BOF samples containing cocaine did not appear at the same degree as cocaine was degrading. These discrepancies could be attributed to the additional degradation of cocaine into EME and ECG (Klingmann et al. 2001). Even though the amount of EME was quantified, the concentrations could not be related to the degradation of cocaine (Appendix D), this because the concentrations of EME were considerably higher (6-35 times) than the initial concentration of cocaine. As mentioned in Chapter 4, EME was detected at high concentrations in OF samples that did not contain this analyte. Therefore, the amount of EME quantified in BOF samples containing cocaine could have been the result of the accumulation of the intrinsic EME and the EME from the degradation of cocaine. The accumulation of EME was no clearly seen probably because of its further degradation into ECG (Klingmann et al. 2001).

5.3.4 Comparison between BOF, SOF and porcine oral mucosa stability

Few differences were seen for the stability of cocaine and cocaine derivatives in BOF, SOF and homogenised oral tissue. Summary of the results obtained for the stability of all analytes in SOF, BOF or tissue is shown in Table 5.3. The results presented in this table show that samples containing AEME and BZE remained stable for a longer period of time in BOF (AEME: two months; BZE: two months) than in SOF (AEME: 15 days; BZE: four days) at room temperature. These differences in the stability of analytes in SOF and BOF could be attributed to the composition of these two matrices. Even though the composition of SOF aims to replace the natural, i.e. neat OF, the SOF may contain different constituents or amount of compounds present in human OF. Additionally, the buffer from the collection device contains exogenous compounds that may contribute to the stability of the analytes in OF, e.g. colourants, preservatives, stabilising salts and surfactants (Lee and Huestis 2014).

Table 5.3 Summary of the results obtained for the stability of cocaine and derivatives in SOF, BOF and porcine oral tissue.

Analyte	Storage Temperature	SOF	BOF	Tissue
AEME	37 ºC	Stable for up to 24 hours	NA	Stable for up to 24 h.
	Room temperature	Stable for up to 15 days. Maximum decrease in concentration of 65% after three months	Stable for up to two months.	NA
	Fridge	Stable for up to two months	Stable for up to two months	NA
	-20 ºC	Stable for up to three months.	Stable for up to two months.	Stable for up to one month.
BZE	37 ºC	• Stable for up to 24 h. • Concentrations increased up to 57% of COC day 0 in 24 h.	NA	• Stable for up to 24 h. • Concentrations increased up to 11% of COC day 0 in 24 h.
	Room temperature	• Stable for up to four days. Maximum decrease in concentration of 68% after three months • Concentrations increased up to 68% of COC day 0 in three months.	• Stable for up to one month. • Concentrations increased up to 72% of COC day 0 in two months.	NA
	Fridge	• Stable for up to two months • Concentrations increased up to 54% of COC day 0 in three months.	• Stable for up to two months • Concentrations increased up to 32% of COC day 0 in two months	NA
	-20 ºC	• Stable for up to three months. • Detected at concentrations <10% COC day 0 over three months.	• Stable for up to one month. • Detected at concentrations <20% COC day 0 over two months	• Stable for up to one month. • Detected at concentrations <5% COC day 0 over one month
COC	37 ºC	Stable for up to 4 h. Maximum decrease in concentration of 64% after 24 h.	. NA	Stable for up to 24 h.
	Room temperature	Stable for up to 4 days. Maximum decrease in concentration of 90% after 3 months.	Stable for up to 4 days. Maximum decrease in concentration of >90% after 2 months	NA
	Fridge	Stable for up to 15 days. Maximum decrease in concentration of 70% after 3 months	Stable for up to 8 days. Maximum decrease in concentration of 83% after 2 months	NA
	-20 ºC	Stable for up to 3 months.	Stable for up to 2 months.	Stable for up to 1 month.
CE	Room temperature	NA	Stable for up to 8 days. Maximum decrease in concentration of 60% after 2 months.	NA
	Fridge	NA	Stable for up to 1 month.	NA
	-20 ºC	NA	Stable for up to 2 months.	NA
NC	Room temperature	NA	Stable for up to 2 months.	NA
	Fridge	NA	Stable for up to 2 months.	NA
	-20 ºC	NA	Stable for up to 2 months.	NA
EME	Room temperature	NA	Stable less than 24 h.	NA
	Fridge	NA	Stable less than 24 h.	NA
	-20 ºC	NA	Stable less than 24 h.	NA

SOF: artificial Oral Fluid, BOF: Buffered Oral Fluid, Tissue: Homogenised porcine oral tissue, AEME: Anhydroecgonine methyl ester, BZE: benzoylecgonine, EME: ecgonine methyl ester, COC: cocaine, CE: cocaethylene, NC: norcocaine, NA: No analysed.

A mean decrease in AEME concentration of 65% was observed on day 90 for SOF samples stored at room temperature. Similar results were reported by Fandiño et al. (2002) where a decrease in concentration of 50% was obtained after 30 days (phosphate buffer PBS pH 7) and five days (human plasma) of storage at room temperature. The more rapid degradation of AEME into AE was attributed to the presence of esterases in the plasma (Fandiño et al. 2002). The increased stability obtained for SOF and BOF compared with the stability reported in PBS could have attributed to the presence of stabilising agents and butyrylcholine esterase inhibitors such as sodium fluoride.

As per AEME, the stability of BZE was prolonged in BOF (one month) than in SOF (four days). This result can be explained by the impact of preservatives present in the BOF as it was previously discussed. Reported stability of BZE in whole blood (McCurdy et al. 1989) and PBS (Kiszka et al. 2000) indicated that BZE in blood remained stable up to 30 days at room temperature. For BZE in PBS, a decreased in concentration of > 60% after 90 days of storage at room temperature was reported. The results presented here for BZE in BOF were similar to the results of preserved whole blood reported by McCurdy (1989) since the analyte remained stable for one month. Unexpectedly, the stability in SOF was shorter (decrease in BZE concentration > 60% after 60 days storage) than the stability reported in PBS, which indicated that fluctuations in the storage temperature (18 °C – 25 °C) influenced the degradation of BZE.

When samples were stored in the fridge, AEME was stable in both matrices for more than two months. This stability was longer than the stability reported in plasma (13 days) (Fandiño et al. 2002) and was attributed to the stabilising agents present in the OF matrices. BZE in BOF showed extended stability (two months) than in SOF (one month), which once again was attributed to the preservatives present in the matrices. The results

for BOF were similar to the stability reported for BZE in PBS (90 days) (Kiszka et al. 2000). However, the stability of BZE in both BOF and SOF was expected to be longer than in the PBS buffer as the latest does not contain any preservative that could have prevented the degradation of BZE.

The stability of AEME and BZE in both matrices was similar as they remained stable for the length of the study (SOF: 90 days and BOF: 60 days). Although no reports were found for the stability of AEME in aqueous solutions, it could be implied from the results reported by Fandiño et al. (2002) that the stability of AEME increases as the storage temperature decrease. Therefore, confirming the results obtained in this study. The stability reported for BZE in PBS (stable up to 90 days at -20 °C) (Kiszka et al. 2000) was in agreement with the results of BZE in BOF and SOF.

Increasing concentrations of BZE as a result of cocaine degradation in OF and other aqueous matrices (PBS, urine, plasma) have been reported by various authors (Kiszka et al. 2000, Ventura et al. 2009, Gao et al. 2010). This tendency was also observed in the present study while evaluating the stability of cocaine in BOF and SOF. Concentrations of cocaine in both matrices were stable up to four days, and concentrations decreased more than 90% from their initial value over the time of the study (SOF: 90 days and BOF: 60 days). The decrease in cocaine concentration was accompanied by an increase in BZE concentration of 72% over a period of 60 days (BOF) and 68% over a period of 90 days (SOF) when samples were stored at room temperature.

Although the formation of BZE correlated with the decrease of cocaine, the sum of the amount of BZE and cocaine was not constant over the time period tested, as it was also observed by other authors (Ventura et al. 2009). Degradation of 26% and 41% were

reported for cocaine in BOF using two different collector devices (Cozart Drug Detection System oral fluid collection device and Intercept oral specimen collection device, respectively) after two to three days of storage at room temperature (Ventura et al. 2009). A decrease of cocaine concentration (mean value) of 11% was obtained on day four of storage using the Concateno Certus collection device. This prolonged stability for the BOF using the Concateno Certus devices could be the result of the impact of stabilising agents in the buffer composition.

The stability of the samples in BOF (eight days for the low and medium concentration and 15 days for the high concentration) and SOF (15 days) was prolonged when samples were stored at 4 °C compared to room temperature. Even though there are no reports of stability in BOF or SOF at this temperature, degradation of <10% was reported for cocaine in neat OF up to four days of storage at 4 °C (Cone and Menchen 1988). Results that were expected as BOF and SOF contained preservatives, e.g. sodium azide (SOF).

At -20 ºC, the stability of cocaine in both matrices was similar as they remained stable for the length of the study (60 days for BOF and 90 days for SOF). This prolonged stability at -20 °C in comparison with stability at 4 °C and room temperature was also reported for cocaine in blood, urine and PBS (Kiszka et al. 2000, 2001). Although cocaine was reported to be stable in the freezer, a small percentage of BZE was observed in PBS and blood samples (< 20%) (Kiszka et al. 2000, 2001). Similarly, a percentage of BZE was seen in the samples of BOF (72% increase in two months) and SOF (68% increase in three months).

Moriya and Hashimoto (1996) reported that samples of blood and other tissues (brain, liver and muscle) containing CE were more stable than the samples containing cocaine.

This result was in agreement with the results obtained in BOF, as CE was stable for up to eight days compare with cocaine that was stable for four days at room temperature. NC on the other hand has been reported to be stable at -20 °C and have a half-life of 43 min at 37 °C in plasma. The reported stability for NC in plasma was in agreement with the stability in BOF when samples were stored at -20 °C (Bouis et al. 1990).

Samples of cocaine and cocaine derivatives (AEME, BZE and cocaine) in homogenised tissue and SOF were stable when stored at -20 ºC. The concentrations of analytes were within ± 20% of the initial concentration value over the time of the study (Tissue: 30 days; SOF:90 days). When samples were kept at 37 ºC, most analytes (AEME and BZE) were stable in both matrices for up to 24 hours. Cocaine for the contrary was less stable in SOF (four hours) than in tissue (24 hours). The increase in the concentration of BZE in homogenised oral tissue as a result of the degradation of cocaine race up to 11% when samples were stored at room temperature. Stability in other tissues than mucosa or tongue tissue was in agreement with the results presented here, as cocaine was stable up to 24 hours of storage at 37 ºC in homogenates of liver, brain and muscle (pH < 7) (Moriya and Hashimoto 1996). Similarly, the results of this study were in agreement with the results reported by various authors (Spiehler and Reed 1985, Kiszka et al. 2001, Rees 2011). No significant changes in cocaine concentration were reported after one and three months in brain samples stored in the freezer (-16 to -20 ºC) (Spiehler and Reed 1985, Kiszka et al. 2001). Cocaine was also reported to be stable when samples were stored at -20 ºC in samples of kidney and liver (Kiszka et al. 2001). Rees (2011) observed changes in cocaine concentration < 15% from day 0 in muscle samples, when samples were stored at -20 ºC over a period of 30 days.

5.4 CONCLUSIONS

The results presented in this chapter indicated that the time of storage and storage temperature influenced the stability (concentration) of cocaine and cocaine derivatives in SOF, BOF and porcine oral tissue. A decrease in drug concentration was seen as the storage temperature increased. The results indicated that the concentration of BZE increased as a result of the degradation, i.e. hydrolysis of cocaine when samples of SOF and BOF were stored at room temperature or in the fridge. This increase in BZE concentration could be used, at least in part, to correct for losses of cocaine in the SOF and OF samples.

The results of the stability of solutions of SOF containing cocaine showed that samples of cocaine remain stable when stored in the freezer (-20 ºC) for as long as 90 days with minimum hydrolysis into BZE (< 10%). When samples were stored in the fridge, the stability of cocaine decreased, and samples were stable up to 15 days. A mean decrease in concentration of up to 70.2% was seen on day 90. Hydrolysis to BZE was seen with concentrations increasing up to 54% of the initial cocaine concentration over the 90 days. When samples were kept at room temperature cocaine in SOF was stable for up to four days, with further degradation (> 90%) seen on day 90. An increase in the concentration of up to 68.4% was seen for BZE on this day (Day 90). Additionally, the stability at 37 ºC indicated that cocaine was stable up to four hours. 24 hours after, a cocaine degradation of > 64% was seen, with an increase in BZE concentration of up to 57%.

Stability of SOF solution containing cocaine derivatives AEME and BZE indicated that the derivatives were stable for up to 90 days when stored at -20 ºC. This stability decreased to 60 and 30 days for AEME and BZE respectively, when samples were stored

in the fridge. Furthermore, when samples were left at room temperature the stability of AEME and BZE was reduced to 15 and four days respectively. After 90 days of storage at room temperature, the AEME mean concentration reduced to 65.4% and the BZE mean concentration was reduced to 67.8%. Stability at 37 ºC indicated that AEME and BZE were stable for up to 24 hours in SOF at all concentrations evaluated.

The Stability conducted in homogenised porcine oral tissue showed that samples containing cocaine and stored in the freezer (-20 ºC) or at 37 ºC were stable for the length of the study (30 days or 24 hours respectively). Maximum BZE concentrations of 11% were quantified in samples stored in the freezer and 37 ºC. Also, AEME and BZE were stable at -20 ºC for up to one month. The Stability at 37 °C demonstrated that samples were stable for up to 24 hours. These results concluded that no changes in concentration of cocaine and AEME should be expected while developing the *in vitro* studies on the release of drugs from oral drug depots into SOF (Chapter 7).

The stability of cocaine in BOF indicated that cocaine degraded into BZE when samples were stored at room temperature or fridge. At room temperature solutions of COC in BOF were stable up to four days. On day 60, a cocaine degradation of > 90% was seen, with an increase in BZE concentration of up to 72%. When samples were stored in the fridge, the stability of cocaine samples were eight to 15 days. A mean decrease in concentration of 83.1%, 75.8% and 31.3% were seen on day 60 at the low, medium and high concentrations respectively. The BZE concentration increased up to 32% of the initial cocaine concentration over the 60 days. Samples of cocaine in BOF stored at -20 ºC were stable for the length of the study (60 days). Cocaine was not significantly hydrolysed into BZE when samples of SOF were stored at -20 ºC. The mean concentration of BZE as a percentage of cocaine on day 0 was < 20%.

Samples of BOF containing cocaine derivatives (AEME, BZE, CE and NC) proved to be stable for at least one month while stored at room temperature, in the fridge or the freezer. Stability in the freezer (-20 ºC) produced longer stability results than in the fridge, as samples were stable for two months. However, some analytes (AEME: medium and high concentrations, BZE: high concentrations and NC all concentrations) remained stable on day 60 while stored in the fridge or at room temperature. Solutions of AEME at the low concentration (5 ng/mL) were stable for up to 11 days at room temperature. Solutions of BZE at the low concentration were stable for 15 to 30 days while stored in the fridge or room temperature.

The stability of EME in BOF at any of the temperatures evaluated indicated that this compound was only stable for few hours (less than 24 hours), with a decrease in concentration of up to 98.5% by day 60. Even though EME was detected, the EME concentrations could not be related to the degradation of cocaine.

In conclusion, samples of BOF, SOF and tissue should be stored at temperatures of -20 °C to preserve the stability of the samples and avoid degradation of cocaine and derivatives from the time of sample collection until sample analysis. SOF samples stored at -20 °C or 4 °C and analysis within two months or 15 days respectively are preferred to maximise result accuracy. Similarly, analysis of BOF samples should be conducted within two months (stored at -20 °C), eight days (stored at 4 °C) or four days (stored at room temperature) from the time of sample collection. Studies using SOF and porcine oral tissue should be conducted at 37 °C within 24 hours, however, subsampling of SOF are recommended at maximum of four hours because of the degradation of cocaine into BZE after this time. Careful consideration should be taken in the interpretation of results from BOF samples as extended times of storage

(transportation) and changes in temperature of storage could lead to degradation of cocaine and cocaine derivatives. The results presented in this chapter aided in the design of the *in vivo* and *in vitro* studies by identifying the best storage conditions and the implications that the length of storage (transportation of samples from Colombia to the UK) could have on the concentration of cocaine and derivatives in the samples.

Chapter 6

CONCENTRATION OF COCAINE AND COCAINE DERIVATIVES IN HUMAN ORAL FLUID SAMPLES FOLLOWING THE INGESTION AND ORAL EXPOSURE TO COCA TEA

6.1 INTRODUCTION

The consumption of coca tea (Mate de Coca) is both legal and socially acceptable in South America (Jenkins et al. 1996, Penny et al. 2009, Rubio et al. 2014). In these countries, the amount of cocaine consumed via coca tea consumption vary depending upon the ethnicity and the reason for consumption, e.g. traditional use or occasional drinking for the relieve of altitude sickness (Engelke and Gentner 1991, Casikar et al. 2010, Biondich and Joslin 2015, Rubio et al. 2015). A common dose of cocaine can vary between 1-15 g of coca leaves per day for individuals that traditionally consume coca leaves by brewing the leaves (Jenkins et al. 1996, Mazor et al. 2006, Rubio et al. 2013, 2015). Because cocaine and other alkaloids are present in small quantities in the plant material, a high amount of coca leaves is required to be consumed in order to obtain psychoactive effects (Rubio et al. 2015). The plant material contains a low amount of cocaine which is of approximately 0.5% (Jenkins et al. 1996, Rubio et al. 2015). For instance, the consumption of coca tea (approximately one gram of coca leaves per cup) produce low oral absorption of alkaloids (< 30%) with no significant stimulant effects (Mazor et al. 2006). These advantages of low cocaine dosages and no psychoactive effects when coca tea is consumed allow the investigation of the kinetics of release of cocaine in oral fluid (OF) using a human *in vivo* safe model.

Even though no psychoactive effects are obtained following oral administration of coca tea, cocaine and metabolites have been detected at high concentrations in biological matrices such as urine, hair and OF following consumption of the coca leaves (ElSohly et al. 1986, Jenkins et al. 1996, Jufer et al. 2000, Mazor et al. 2006, Strano-Rossi et al. 2008, Reichardt 2014, Rubio et al. 2015). Initially, ElSohly et al. (1986) reported the

detection of benzoylecgonine (BZE) in urine following the ingestion of a cup of coca tea (Health Inca tea) for up to 29 hours post consumption using a cut-off of 100 ng/mL. Jenkins et al. (1996) confirmed that cocaine and metabolites (BZE and ecgonine methyl ester – EME) could be detected in urine after coca tea had been consumed for up to 20 hours (cut-off value of 6.25 ng/mL).

Strano-Rosi et al. (2008) reported peak concentrations for cocaine, BZE and EME in OF following the consumption of Peruvian mate de coca (1g coca leaves) of 23, 27 and 15 ng/mL, with times of last detection of 4.3 hours for cocaine, 11 hours for BZE and one hour for EME. In a different study, Jenkins et al. (1995) and Cone et al. (1997) described that cocaine and derivatives were detected in OF at concentrations significantly higher than in blood (16–505 ng/mL for OF and 0.4–1.9 ng/mL for blood) following smoking of crack cocaine and that this initial high concentration was the result of oral contamination. Oral contamination was reported to disappear over the first 30–120 minutes after oral administration. Furthermore, these drugs were detected over an extended period of time following smoking (40 g dose: eight hours and 42 g dose: 12 hours). This excretion of cocaine and metabolites in OF was attributed to passive diffusion of analytes from the systemic circulation and drug depots.

Reichardt (2014) also reported the detection of high concentrations of cocaine and cocaine metabolites (BZE, anhydroecgonine methyl ester AEME and cocaethylene CE) in OF samples collected from volunteers that had ingested coca tea. Maximum concentrations of cocaine and BZE were reported at 2729 (14-8595) ng/mL and 174 (11-452) ng/mL immediately after the ingestion of the tea. This study showed detection of both cocaine and BZE in OF over one-hour post consumption of the tea, suggesting that cocaine and cocaine derivatives were released from buccal tissues into OF over time. The

study also revealed a number of unexplained artefacts relating to the random detection of AEME and CE in OF samples collected after coca tea consumption.

Since there is not a clear understanding on the impact of drug depots in the excretion of cocaine into OF, it was important to further enhance our understanding of cocaine absorption and elimination from oral/buccal tissues. In order to achieve this, OF samples were collected following the consumption or swirling of a cup of coca tea from human volunteers in a controlled *in vivo* study. The study was undertaken in collaboration with the National University of Colombia from Bogota - Colombia where consumption of coca tea is legal and a regular social custom. Analysis of the coca tea was conducted using a validated method (Chapter 4) to evaluate the presence of cocaine, AEME, BZE, CE and nor-cocaine (NC) in the plant material and coca tea. These cocaine derivatives have been previously reported in OF following administration of cocaine, crack-cocaine and coca tea (Jenkins et al. 1995, Cone et al. 1997, Jufer et al. 2000, Lewis et al. 2004, Cardona et al. 2006, Reichardt 2014). AEME, NC and CE were also reported to be randomly distributed throughout collected OF samples following consumption of coca tea (Reichardt 2014). Therefore, it is of great importance to evaluate whether other cocaine derivatives than BZE such as AEME could be detected on OF after ingestion of coca tea as this could have an impact in OF drug testing. AEME is commonly used as a biomarker for detection of “crack” cocaine and detection of this analyte in OF could wrongly indicate previous use of “crack” cocaine, resulting in legal prosecution of the individual.

This study was also used to investigate whether the equivalence in concentration of cocaine and cocaine derivatives in OF samples when samples are collected from different sides of the mouth using one or two OF collection devices.

6.1.1 AIM AND OBJECTIVES

6.1.1.1 *Aim:*

This chapter aimed to contribute to the knowledge of the kinetics of cocaine and its metabolites in OF following ingestion or swirling of a cup of coca tea.

6.1.1.2 *Objectives:*

- Measure the amount of cocaine and cocaine derivatives present in a cup of coca tea prepared with coca tea bags from the Nasa community (Colombian community).
- Determine concentration profiles of cocaine, BZE, AEME, EME, CE and NC in oral fluid following the ingestion or swirling of a cup of coca tea.
- Evaluate times of last detection for cocaine and cocaine derivatives in OF following the ingestion or swirling of a cup of coca tea.
- Assess differences in concentration profile and time of last detection between participants that ingested or swirled a cup of coca tea.
- Calculate significant differences between the concentration of cocaine and cocaine derivatives from OF samples collected from different sides of the mouth.
- Evaluate population-level differences in the kinetics of cocaine and metabolites from OF samples from participants that have consumed coca tea.

6.2 METHODS

6.2.1 Materials

Drug standards and reagents used for the analysis of buffered oral fluid (BOF) were purchased as described in Section 4.2.1.

Oasis mixed-mode cation exchange (MCX) micro-elution plates (Waters, Manchester, UK) MS and Alere™ Concateno Certus® OF collection devices were donated by Alere™ Toxicology. The Oasis micro-elution plates were used for sample preparation prior to quantitative analysis by LC-MS as described in Section 4.2.9.

The coca tea was prepared using coca leaves from the Nasa community (Colombian indigenous tribe), commercialised as tea bags under the name "Coca Nasa *Nasa Esh's – Aromatica de Coca*". These coca leaves were grown, recollected and dried using traditional procedures, no chemical and/or preservatives were added or used in the process; leaves were sun-dried. The coca leaves commercialised by Nasa community belong to the *Erythroxylum novogranatense* var. *novogranatense* (*E. novogranatense* var. *nov)*. Tea bags from the same batch were used to prepare the tea. The tea bags were purchased from Coca Nasa *Nasa Esh's* (Cauca – Colombia).

6.2.2 Instrumentation

Analysis of BOF samples was carried out using a LC-MS/MS system consisting of a tandem quadrupole mass spectrometer coupled to a Waters Acquity UPLC® system. Details on the LC-MS systems were described in Section 4.2.2.

6.2.3 Preparation of coca tea - Dosage

A cup of coca tea was prepared by infusing two coca tea bags (1.04 ± 0.08 g) in 200 mL boiling water (91 ºC; 2640 m). The tea was ready to drink after leaving the tea bags infusing for eight minutes without any stirring. The coca tea was prepared based on customary dosage (approximately one gram) and procedures (Jenkins et al. 1996, Mazor

et al. 2006). Two coca tea bags were used as the amount of coca leaves per tea bag present in the Coca Nasa *Nasa Esh's* was 0.52 ± 0.04 g (measurement based on 30 tea bags). Eight minutes were chosen based on the results presented by Jenkins et al. (1996), who demonstrated there were not significant differences between the amount of cocaine extracted after infusing coca leaves for eight and nine minutes.

6.2.4 Pilot study

OF samples were collected from five participants (male and female age ranging 30-80) over a period of six hours following the ingestion of a cup of coca tea (prepared as per Section 6.2.4). The results of this study indicated that cocaine and derivatives were no longer detected in OF after three hours of ingestion. Thus, a period of four hours was selected for the main study as this time would allow the monitoring of cocaine and derivatives in OF until no analytes could be detected in the OF and minimising the time for the volunteers to participate in the study.

6.2.5 Study population

The study was conducted with undergraduate students (male and female with age ranging 18-33) from the Department of Pharmacy of the National University of Colombia in Bogota – Colombia.

The number of volunteers (sample size) required in this study was elucidated based on the results obtained from a pilot study (as per Section 6.2.4). The sample size for the study was 30 participants. This value was calculated using the Equation 6.1 and the following

values: mean cocaine concentration of 160 ng/mL, imprecision of ± 20%, standard deviation of 16%, significance level of 0.05 and power of 80% (Crawley 2005).

$$n = \frac{0.8 \times s^2}{\partial^2} \qquad \text{Equation 6.1}$$

Where *n* is the sample size, *s* the variance and ∂ the difference that want to be detected with a probability of 0.8.

The inclusion criteria were being healthy volunteers, male and woman with age 18 years or above, be able to give written inform consent and able to understand and complete a questionnaire (Appendix E) and able to provide OF samples after ingestion or swirling of a cup of coca tea. The participants were excluded from the study if they did not give written consent or if they acknowledge had consumed cocaine, crack-cocaine or coca tea in the three days before the study.

All subjects who met the inclusion and exclusion criteria were invited to participate in the study. A questionnaire was obtained from all participants along with informed consent for the collection of OF samples. Details of the recruitment process were explained in the protocol of the study and the volunteer information form as indicated in Appendix E.

6.2.6 Ethics

This study was approved by the ethics committees of Bournemouth University and the National University of Colombia - Bogota. The approved questionnaire, protocol, participant information form and volunteer information form are shown in Appendix E.

6.2.7 Collection of control oral fluid samples

Samples of control OF used for the preparation of calibrants, QCs and blanks were collected from six healthy volunteers (male and female with ager ranging 25-35 years old) using the Concateno Certus® devices as described in Section 4.2.5.

6.2.8 Collection of oral fluid samples

This study was conducted as described in the protocol of the study (Appendix E). Initially, verbal information about the study was given to all participants at the beginning of the study. The 30 participants were divided in two groups (Group A and B) using randomised allocations. Once consent was received from all participants a single sample of OF was collected from each participant (pre-dose sample) by active sample collection following manufacture guidance (Section 4.2.5). Subsequent OF samples were collected following the ingestion or swirling of a cup of coca tea:

Group A comprised 15 participants each of whom drank and swallowed one cup of coca tea within a maximum period of 10 minutes. Following ingestion, all participants provided OF samples at 10, 20, 30, 60, 120, 180 and 240 minutes using one Concanteno Certus device at each time point (active sample collection). OF was collected as described in the protocol of the study (Appendix E) and Section 4.2.5.

Group B comprised 15 participants each of whom mimicked the drinking of a cup of coca tea by sipping, holding and swirling tea in the mouth for 20-30 seconds and then spitting out the content. This process was repeated several times to complete the sipping-spitting of a cup of coca tea for a maximum period of 10 minutes until all the tea had been

used. Following swirling, all participants provided two simultaneous OF samples at 10, 20, 30, 60, 120, 180 and 240 minutes using two Concanteno Certus devices at each time point to observe whether differences were obtained from different sides of the mouth. One device was located on the right side of the mouth (R) and the second device on the left side of the mouth (L). The devices were placed between the inner side of the cheek and the teeth. OF was collected as described in the protocol of the study (Appendix E) and Section 4.2.5.

The length of the study (240 minutes) was selected based on the results obtained from the pilot study (five volunteers).

6.2.9 Storage of buffered oral fluid samples

All absorbent pads (containing 1 mL OF) were left at room temperature (10-20 ºC) into the respectively labelled tube (which contained the extraction buffer) for 24 hours to ensure a complete extraction of the analytes into the buffer (The mean recovery from the collection pad into the buffer was 97 ± 1.4% for all analytes). Subsequently, each collection pad was centrifuged at 3000 rpm for 5 min to recover all BOF. All samples were weighted before and after centrifugation to accurately measure the amount of OF collected in each sample. The samples were then split in two and stored at -20 °C (2-8 days) until shipment to the UK. During transportation from Colombia to the UK (seven days) the storage conditions could not be monitored and therefore variations in the concentration of analytes in OF were expected. After the samples arrived in the UK, they were stored at Alere Toxicology at -20 °C until analysis.

6.2.10 Quantitative analysis of buffered oral fluid samples

All buffered samples were extracted and analysed for AEME, BZE, COC, CE, NC and EME at Alere Toxicology using a validated LC-MS/MS method. Details of the SPE extraction and LC-MS/MS method were given in Chapter 4. All samples were analysed by the researcher using calibrators and QCs prepared on the day of analysis.

All results were reported as concentration of analyte in original "neat" OF rather than in BOF. To obtain the concentration in neat OF, the concentrations of analyte in BOF were corrected by the dilution factor of the collection device (multiplying by four).

6.2.10.1 *Quantitation of cocaine and cocaine derivatives in coca tea*

Table 6.1 shows the amount of cocaine and cocaine derivatives present in a cup of coca tea Coca Nasa *Nasa Esh's*. These values were obtained from the analysis of 30 separate teacups prepared as mentioned in Section 6.2.3. All samples of coca tea were diluted (100x) in control buffered oral fluid before storage. Buffered samples were finally extracted and analysed as described previously.

Table 6.1 Amount of cocaine and cocaine derivatives present in a cup of coca tea (200 mL) using Coca Nasa *Nasa Esh's* tea bags.

Analyte	Amount (mg) with $n = 30$	
	Mean ± SE	Range
AEME	1.2 ± 0.03	0.9-1.5
BZE	0.8 ± 0.04	0.4-1.2
COC	6.7 ± 0.20	5.2-9.0
CE	-	-
EME	87.2 ± 5.02	40-134
NC	-	-

AEME: Anhydroecgonine methyl ester; BZE: Benzoylecgonine; COC: Cocaine; CE: Cocaethylenene; EME: Ecgonine methyl ester; NC: Norcocaine; *n*: Number of samples; SE: Standard error

In order to confirm the presence of AEME in the plant material, a methanolic extract of the Colombian coca tea bags (1.05 g coca leaves) was analysed following the method described by Jenkins et al. (1996). The extraction (using 200 mL methanol and mechanical shaking for 48 hours) was conducted at room temperature to avoid the formation of AEME from thermal exposure of cocaine. All methanolic extract were diluted (100x) in control buffered oral fluid before storage. Buffered samples were finally extracted and analysed as described previously.

6.2.11 Data analysis

The data was analysed based on analytical cut-off concentrations in OF (Limit of quantification LOQ) and the European Workplace Drug Testing Society (EWDTS) cut-off. The EWDTS recommend a cut off concentration of 8 ng/mL for confirmation of cocaine and BZE in neat OF (EWDTS 2015).

IBM SPSS Statistics version 23 and Microsoft Excel Version 15.33 were used for statistical analysis. Nonparametric Spearman's test was used for correlation analysis. Mann-Witney (U) test and Wilcoxon (W) test were used to compare the data. These tests were chosen after verifying the absence of normal distribution (Kolmogorov-Smirnov normality test) and homogeneity (Levene's test) of variance. Results with 2-tailed $p_s < .05$ were considered significant.

6.3 RESULTS AND DISCUSSION

6.3.1 Amount of cocaine and cocaine derivatives in a cup of coca tea

Quantitative analysis of the coca tea prepared with tea bags "Coca Nasa *Nasa Esh's"* as described in Section 6.2.10.1 (Table 6.1) demonstrated that the principal alkaloid present in the coca tea was EME (87.2 mg) followed by cocaine (6.7 g), AEME (1.2 mg) and BZE (0.8 mg). These results were different from the amounts reported by other authors on the analysis of alkaloids in coca tea prepared with coca leaves from Peru (EME 2.0 mg, COC 4.8 g, AEME 0.01 mg and BZE 0.8 mg) and Bolivia (EME 2.3 mg, COC 4.8 g, AEME 0.06 mg and BZE 0.2 mg) (Jenkins et al. 1996). The coca tea prepared by Jenkins et al. was prepared under similar conditions as the ones used in this chapter using approximately 1 g of coca leaves, 180 mL water (94 °C) and nine minutes infusion time. Summary of the results obtained in this chapter and the results reported by Jenkins et al. (1996) are shown in Table 6.2. In this table the amount of analyte presented in the coca tea was corrected for 200 mL coca tea.

Table 6.2 Amount of cocaine and cocaine derivatives present in a cup of coca tea prepared with coca leaves from Colombia, Peru* and Bolivia*. Coca tea was prepared using approximately 1g of coca leaves.

Analyte	Mean amount of analyte (mg)**		
	Colombian coca tea (*n* = 30)	**Peruvian coca tea* (*n* = 30)**	**Bolivian coca tea* (*n* = 30)**
AEME	1.24	0.01	0.06
BZE	0.83	0.78	0.17
COC	6.75	4.76	4.81
CE	-	-	-
EME	87.2	1.98	2.28
NC	-	-	-

AEME: Anhydroecgonine methyl ester; BZE: Benzoylecgonine; COC: Cocaine; CE: Cocaethylenene; EME: Ecgonine methyl ester; NC: Norcocaine; *n*: Number of samples. * Data reported by Jenkins et al. (1996). ** mg per 200 mL coca tea using approximately

Comparison between the results presented in Table 6.2 indicated that the amount of cocaine and EME were higher (1.2 times for COC and 40 times for EME) in the Colombian tea than the Peruvian or Bolivian tea. The amount of BZE was similar (< 4% difference) between the Colombian tea and the Peruvian tea.

The high amount of EME obtained in the coca tea (87±5 mg) could be the result of thermal degradation of cocaine in the tea (Klingmann et al. 2001) and/or to its extraction from the plant material, i.e. coca leaves. However, based on the results of other authors (Jenkins et al. 1996, Casale et al. 2014) it can be suggested that the primary source of EME in the tea (Coca Nasa *Nasa Esh's*) was the extraction of EME from the plant material and not the thermal degradation of cocaine. Jenkins et al. (1996) considered that thermal degradation of cocaine into EME was less likely to occur during the preparation of the tea than its presence in the plant material. This was explained by the fact that EME and BZE were present in the tea bags and were not a product of the hydrolysis of cocaine (Jenkins et al. 1996). Casale et al. (2014) reported the presence of EME in extracts of 15 different species of the coca plant including *E. novogranatense* var. *nov*.

No CE or NC was detected in the coca tea. These results were in accordance with the results reported by Jenkins et al. (1996), who did not detect any of these analytes in Peruvian or Bolivian coca tea, although Casale et al. (2014) reported the presence of NC but not CE in organic extracts (e.g. toluene, methanol and chloroform) of the coca plant (*E. novogranatense* var. *nov*.). This result was unexpected as NC is an active metabolite formed from the enzymatic degradation of cocaine in the liver (Poon et al. 2014). The qualitative analysis described by Casale et al. (2014) indicated that NC was present at considerable low amounts (less than ten times the peak high of COC or EME) in organic extracts of coca leaves. These results suggested that NC could be extracted during the

preparation of a cup of coca tea but the amount of NC could be at undetectable concentrations.

AEME has been detected in coca tea and the coca plant by few authors (Jenkins et al. 1996, Rubio et al. 2015, 2016) however, all authors attributed its presence as an artefact product of the GC-MS analysis or its production from cocaine as a result of thermal exposure during extraction. The results presented in Table 6.2 indicated that AEME was present in the Colombian tea at high amounts (1.24 mg/g coca leaf) compared with the amounts present in coca tea prepared with Peruvian or Bolivian coca tea bags (0.01-0.07 mg/g coca leaf) (Jenkins et al. 1996). This difference in concentration could be attributed to differences in the species of the coca leaf and analytical procedures. Even though the authors did not report the species of the coca plant in Peruvian and Bolivian coca tea, it has been reported that the predominant species of coca leaf in Peru and Bolivia belong to the *Erythroxylum coca* (Plowman 1979, Casale et al. 2014) differing from the species found in the Colombian coca tea that belonged to the *E. novogranatense* var. *nov*.

The presence of AEME in the coca tea as an artefact product of the detection process (LC-MS method) was discarded as AEME was not detected when standards of cocaine were evaluated during the LC-MS method development and validation (Chapter 4). The results of the analysis of AEME from the methanolic extract (extraction conducted at room temperature) indicated that 12.2 ± 0.1 mg of AEME was present in one gram of coca leaves and that only 38% of the total AEME present in the plant was extracted during the preparation of the tea. Further confirmation of AEME concentration in the plant material is required as the methanolic extraction of AEME and the other analytes was not optimised and higher amounts of AEME could be present in the coca leaves.

The high amount of AEME present in the coca leaves indicated that AEME concentration in the coca tea was most likely to be the result of extraction from the plant material than its formation from thermal exposure during the preparation of the tea. This assumption could be supported by the fact that other analytes such as BZE and EME were not produced during tea preparation (Jenkins et al. 1996). Nonetheless, more studies need to be conducted in order to confirm that AEME was not formed during tea preparation. The fact that AEME was detected in coca tea was of great importance as detection of this analyte in OF samples could wrongly indicate recent consumption of crack cocaine. This outcome could have potential impact on OF drug testing as positive results could be obtained for AEME after consumption of coca tea. Although OF is not yet used in countries where coca tea is traditionally used, there have been reports that coca tea can be purchased in several countries such as the USA where OF testing is implemented and where the importation of coca leaves is illegal (Mazor et al. 2006).

6.3.2 Demographics and user experiences

A total of 30 participants were screened for cocaine and metabolites following the ingestion (Group A; n = 15) or swirling (Group B; n = 15) of a cup of coca tea. Table 6.3 summarises the demographics obtained for the participants. 29 participants were undergraduate students (National University of Colombia) with age ranging 18-25 years. Only one participant from the Group B was aged 26-33 years. The majority of participants that ingested the coca tea were males (60%), whereas the majority of participants that swirl the tea were female (60%). Median values for height, weight and BMI were 1.7 m, 61.0 Kg and 21.4 Kg/m^2 respectively. Most BMI values (83%) within the range 18.5-24.99 Kg/m^2 were considered normal according to the World Health Organisation (WHO). Two participants (BMI of 15.7 and 17.1 Kg/m^2 of BMI were underweight (WHO

Underweight BMI: < 18.5 Kg/m^2) and three participants (BMI of 26.1, 26.4 and 26.8 Kg/m^2) were pre-obese (WHO Pre-obese BMI: 25.0-29.9 Kg/m^2). A statistical Wilcoxon (W) test indicated that there were no significant differences in the height, weight and BMI between the participants from Group A (Ingestion) and B (Swirling) (Height: W (30) 204, p = 0.25; weight: W (30) 216, p = 0.49; BMI: W (30) 231, p = 0.97). Only eight participants reported to be smokers (Group A - Ingestion: 20%; Group B - Swirling: 33%).

Table 6.3 Demographics of the participants that ingested or swirled a cup of coca tea.

Characteristic	Group A - Ingestion	Group B - Swirling
n	15	15
Age	18-25	18-33
Gender	Female 40% (n = 6) Male 60% (n = 9)	Female 60% (n = 9) Male 40% (n = 6)
Height (m)*	1.74 (1.52 – 1.8)	1.65 (1.5 – 1.8)
Weight (Kg)*	65 (45 – 78)	58 (40 – 74)
BMI (Kg/m^2)*	21.6 (15.7 – 26.4)	21.0 (17.1 – 26.8)
Smokers	20% (n = 3)	33% (n = 5)

n: Number of participants; * Mean values and range

Most participants (67%) were originally from Bogota – Colombia and the remaining 10 participants (33%) were from the following cities in Colombia: Barrancabermeja, Cúcuta, Sogamoso, Villeta, Zipaquirá, Duitama, Garagoa, Ipiales, Tunja and Valencia.

Eleven (37%) of the 30 participants reported previous consumption of coca tea. the aforementioned 11 participants reported consumption of coca tea less than once a month in the city of residence. The tea was primarily consumed in the afternoon (n = 6, 55%), followed by evening (n = 3, 27%) and morning (n = 1, 9%). The tea was reported to be prepared using one bag (n = 7, 64%), two tea bags (n = 3, 27%) or leaves (seven leaves per teacup) (n = 3, 27%) and mixed with sugar (n = 9, 80%) or drunk on its own (n = 2, 20%). From five studies reported on the use of coca tea (Engelke and Gentner 1991,

Jenkins et al. 1996, Mazor et al. 2006, Penny et al. 2009, Zavaleta 2015) only two studies reported the consumption of coca tea with sugar (Penny et al. 2009, Zavaleta 2015). None of these reports mentioned the amount of coca leaves used (dose) or the time at which the coca tea was consumed.

The answers reported by the 30 participants on the questionnaire also allowed the evaluation of the clinical and psychological effects of consumption of coca tea, as well as the reason for consumption of the tea for the study population. These data were summarised in Figure 6.1. Figure 6.1-A shows the reason for consumption of coca tea given by the participants that had previously consumed coca tea (n = 11). The reasons given were as follow: consumption for traditional reasons (n = 2), altitude sickness (n = 3), test or flavour (n = 2), interest (n = 1), curiosity (n = 3), to increase energy (n = 1) or elevate mood (n = 1). These results were in agreement with few publications that associated the consumption of coca tea to tradition, relieve of symptoms for altitude sickness or nutritional reasons such as elevated energy (Penny et al. 2009, Casikar et al. 2010, Biondich and Joslin 2015, Zavaleta 2015).

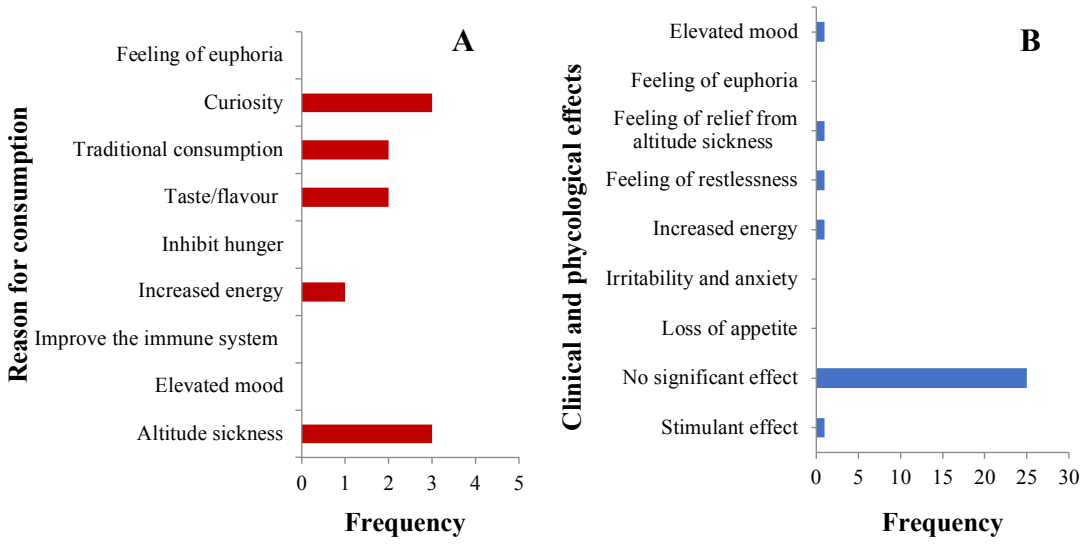


Figure 6.1 (A) Reason for consumption of coca tea. (B) Clinical and psychological effects after consumption of coca tea.

On the other hand, Figure 6.1-B, showed the experiences of the participants (n = 30) following the consumption of coca tea. Most participants (n = 25, 83%) reported 'no-significant effects' following consumption of coca tea. The remaining five participants reported have effects of elevated mood, feeling of relieve from altitude sickness, feeling of restlessness, increased energy and stimulant effect. Three of the five participants that reported effects following consumption of coca tea were the same participants that reported consuming coca tea for the same effects (altitude sickness, increase energy or elevate mood). The results obtained were in agreement with previous reports (Penny et al. 2009, Biondich and Joslin 2015) which described that coca tea is mostly consumed in the Andean region for traditional reasons as well as for the effects that this could offer to people including antifatigue effect, suppression of appetite, nutritional factors and relieve to altitude sickness. The primary reason for consumption of coca tea was therefore related traditional reasons with no significant effects obtained following the ingestion of a cup of coca tea, however effects such as those described previously can be obtained for a small percentage (17%) of the population.

The results of the questionnaire also revealed that 80% of the participants did not expect to have any effect following consumption of coca and 93% did not considered coca tea a psychoactive substance. These results were in agreement with the report of 83% of the participants not reporting any significant effect after the intake of the tea (Figure 6.1-B), confirming that consumption of coca leaves is not considered an addiction in regions where it is customarily used as mentioned by Rubio et al. (2014).

6.3.3 Concentration of cocaine in oral fluid following the ingestion (Group A) and swirling (Group B) of a cup of coca tea

The concentration of cocaine in OF samples in the control samples (pre-dose samples) ranged 1-17 ng/mL for all participants. The concentration range for the group that ingested the tea (Group A) was 1-9 ng/mL. The concentration range for the group that swirled the tea (Group B) was 1-17 ng/mL. Summary of cocaine concentrations in OF following the ingestion or swirling of coca tea is presented in Appendix E Table 3.

The results showed that two out of 15 participants tested positive (based on the EWDTS cut-off) for the presence of cocaine in the control sample even though all participants reported not have consumed coca tea or cocaine 72 hours before the study. The EWDTS guidelines for cocaine confirmatory testing in OF established a cut-off concentration of 8 ng/mL for cocaine (EWDTS 2015). As per most voluntary studies, consumption of cocaine or coca tea prior to the study could not be ensured. Table 6.4 shows the descriptive statistics of cocaine concentration at each collection time.

Table 6.4 Cocaine concentration in neat OF following the ingestion or swirling of a cup of coca tea.

Collection time (min)	Concentration Group A – Ingested (ng/mL) (n = 2)*					Concentration Group B - Swirled (ng/mL) (n = 2)*				
	Mean	Median	SD	Min	Max	Mean	Median	SD	Min	Max
0	2	2	2	1	9	3	2	4	1	17
10	1248	1215	853	58	2993	2553	2569	1012	274	4643
20	543	449	564	14	2168	1168	1254	755	68	2855
30	225	105	283	4	1082	483	206	554	11	2172
60	61	31	66	3	203	78	45	73	3	276
120	16	8	35	1	140	9	4	13	1	48
180	7	4	11	1	42	4	4	2	2	9
240	6	3	9	1	36	2	2	1	0	6

Group A: Participants ingested one cup of coca tea (data collected using one collection device at each time point). Group B: Participants swirled a cup of coca tea (data collected using two collection devices at each time point). A: OF sample collected from the right side of the mouth. B: OF sample collected from the left side of the mouth. SD: Standard deviation. LOQ: 0.5 ng/mL cocaine in buffered oral fluid; LOQ in neat OF was 2 ng/mL (the dilution factor from collection device was 4) * Each sample was analysed in duplicate and values are presented as mean values.

The two participants that tested positive were Participant P3 from Group A with 9 ng/mL and Participant P29 from Group B with 17 ng/mL. Mean and median values were below the EWDTS cut-off for Group A and Group B. After excluding the positive pre-dose samples, the mean and median values did not change significantly (Group A - Ingestion: Mean = 1.7, Median = 1.6; Group B - Swirling: Mean = 1.7, Median = 1.6). Maximum cocaine concentrations of negative pre-dose samples were 4.8 and 3.0 ng/mL in OF for Group A and B respectively excluding the positive pre-dose samples.

Figure 6.2 illustrates the concentration profile of cocaine in OF for a period of four hours following ingestion (Group A) or swirling (Group B) of coca tea. Furthermore, Table 6.5 summarises the maximum concentration (Cmax), times of maximum concentration (T_{max}), time of last detection ($T_{0\text{-last}}$) and area under the curve ($AUC_{0\text{-last}}$) for both groups. Mean maximums cocaine concentration (C_{max}) in OF of 1248 and 2534 ng/mL for Group A (Ingestion) and B (swirling) respectively were observed in the first sample collected after 10 minutes following ingestion and swirling of the tea. Based on the EWDTS (EWDTS 2015) and LOQ cut-offs all participants reported positive for cocaine in the sample collected after 10 minutes of the ingestion or swirling of coca tea.

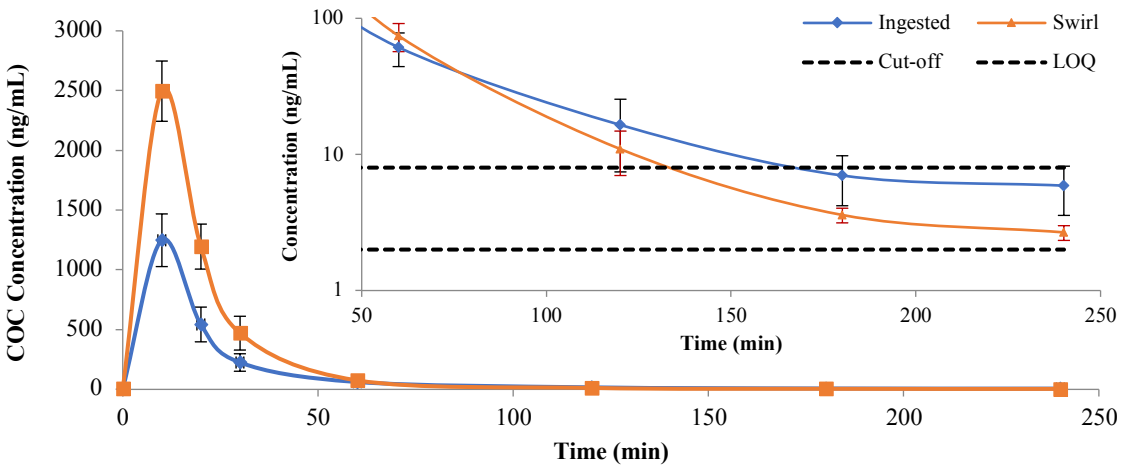


Figure 6.2 Mean cocaine concentrations in OF following the ingestion (n = 15, 120 samples) or swirling (n = 15, 240 samples) of a cup of coca tea. Bars are standard deviation. Data illustrated in full scale and with a zoom into the low concentration region. Dotted lines indicate the following cut-off concentrations: Limit of quantification (LOQ) (2 ng/mL), EWDTS (8 ng/mL).

Table 6.5 Maximum concentration, time at maximum concentration, detection window and area under the curve for cocaine in OF following the ingestion (Group A) or swirling (Group B) of a cup of coca tea.

Group A - Ingested					Group B - Swirled				
Participant	**C_{max} (ng/mL)**	**T_{max} (min)**	**T_{last} (min)**	**$AUC_{0\text{-}last}$ (ng/mL/min)**	**Participant**	**C_{max} (ng/mL)**	**T_{max} (min)**	**T_{last} (min)**	**$AUC_{0\text{-}last}$ (ng/mL/min)**
P1	1886	10	240	594	**P16**	2862	10	120	39496
P2	1317	10	120	399	**P17**	2533	10	120	27306
P3	1630	10	180	350	**P18**	2674	10	180	73463
P4	1242	10	120	543	**P19**	2496	10	180	44924
P5	2137	10	>240	1009	**P20**	1388	10	120	19249
P6	737	10	180	207	**P21**	4609	10	180	164380
P7	2992	10	120	1245	**P22**	3420	10	240	92801
P8	1115	10	120	297	**P23**	2479	10	60	30241
P9	1215	10	120	273	**P24**	2473	10	180	57191
P10	2359	10	180	510	**P25**	3667	10	120	52353
P11	207	10	180	92	**P26**	1875	10	120	15860
P12	505	10	180	111	**P27**	305	10	60	9125
P13	58	10	60	17	**P28**	3064	10	120	76494
P14	1176	10	180	469	**P29**	2212	10	120	50888
P15	138	10	60	41	**P30**	1844	10	120	42024
Mean	1248	10	158	410		2527	10	137	53053
Median	1215	10	180	350		2496	10	120	44924
SD	853	0	55	346		1003	0	48	38704
Min	58	10.0	60	17		305	10.0	60	9125
Max	2992	10.0	240	1245		4609	10.0	240	164380

C_{max}: Maximum concentration; T_{max}: Time at maximum concentration; T_{last}: Time of last detection at EWDTS cut-off (8 ng/mL); $AUC_{0\text{-}last}$: Area under the curve. SD: standard deviation.

A rapid decrease in concentration in both groups was observed over the first hour to a mean concentration of 61 and 77 ng/mL for Group A - Ingestion and Group B - Swirling respectively. After two hours, 47% (Group A-Ingested) and 33% (Group B-Swirling) of the population still reported positive at both cut-offs. Mean concentration values at two hours were 7.0 ng/mL for Group A and 3.9 ng/mL for Group B. The time of last detection at the EWDTS cut-off for Group A was three hours and for Group B - Swirling was two hours (Table 6.5). Two participants (13 % of the population) from the Group A - Ingested gave positive results for cocaine during the length of the study (four hours). Only one participant tested positive in Group B – Swirling three hours after having swirled the tea but his concentration in OF decreased below the cut-off after four hours. The time of last detection at the assay's LOQ was longer for both groups. More than 60% of the

population (Group A - Ingested: 80%; Group B - Swirling: 60%) had detectable concentrations of cocaine in OF after four hours.

Figure 6.2 shows that higher concentrations in OF were obtained when the coca tea was swirled (Group B) than when it was ingested (Group A). A Mann-Whitney (U) test confirmed that there were significant differences between the C_{max} (U = 33, p = 0.001) and $AUC_{0\text{-}last}$ (U = 0, p < 0.001) among both groups. Similarly to C_{max}, there were significant differences in cocaine concentrations between both groups at 20 minutes (U = 53, p = 0.013 Z = -2.5). After 30 minutes there were no significant differences between both groups (p > 0.05).

Although the statistical analysis indicated that there were not significant differences between both groups, it was noticed that concentrations of cocaine in OF in the group that ingested the tea (Group A) were greater than the group that swirled the tea (Group B) in samples collected at or after 120 minutes of ingestion/swirling. Mean concentration of cocaine in OF was 16 ng/mL for Group A and 9 ng/mL for Group B at time point 120 minutes. This increase in cocaine concentrations for Group B (Swirling) after 120 minutes of ingestion/swirling resulted in a higher T_{last} (Mean: 158 min; Median: 180 min) than the T_{last} for Group A (Ingestion) (Mean: 137 min; Median: 120 min). A possible explanation for these results could be the idea that during the drinking of the tea (Group A) two processes took place: (1) the formation of drug depots in the oral tissues, i.e. absorption of cocaine in the oral cavity and its subsequently release into oral fluid and (2) the elimination of cocaine from the systemic circulation into oral fluid. Since the participants that swirled the tea (Group B) did not swallow the tea, only the elimination of cocaine from drug depots could have taken place. For instance, it is possible that the contribution

of cocaine from the systemic circulation resulted in an increase in cocaine concentration in Group A – Ingestion.

Elevated concentrations of cocaine in OF were expected to be present in the OF immediately after ingestion/swirling of the tea because of the contamination of the oral cavity with the excess of coca tea (Jenkins et al. 1995, Cone et al. 1997, Huestis and Cone 2004). The differences in concentration of cocaine in OF between the two groups during the first 20 minutes after ingestion/swirling were attributed to differences in the amount cocaine absorbed in oral tissues, i.e. formed oral depots, which were subsequently released into the OF. The results implied that cocaine was rapidly absorbed in the oral tissues forming drug depots even after a short period of exposure to the coca tea (time spent on sipping and swallowing or sipping and spitting out the coca tea).

During the development of the study, it was noticed that participants that swirled the tea (Group B) held it in the mouth for a longer period of time (before spitting it) than the participants that sipped the tea and then ingested it (Group A). Hence, it was considered that these different times of exposure to the tea could have contributed to the differences in the number of drug depots formed in the oral cavity which reflected in the concentration of cocaine in OF. In the present study, all participants ingested or swirled an equal dose of 1.04 g of coca leaves (6.8 mg COC – see Table 6.2) over a maximum period of 10 minutes.

Similar cocaine concentration profiles in OF were reported by others authors regarding the detection of cocaine in OF following oral administration of cocaine: drinking dose of 3 mg, smoke doses of 40 and 42 mg or intranasal dose of 32 mg (Jenkins et al. 1995, Cone et al. 1997, Strano-Rossi et al. 2008). In these studies, the initial high cocaine

concentration in OF was reported to rapidly disappeared within a period of 0.5-3 hours after oral or intranasal drug administration. The authors suggested that rapid dissolution of cocaine in saliva following oral exposure to the drug allowed the effective clearance by swallowing and that detection of cocaine in OF after two hours appeared to be determined primarily by passive diffusion from the blood into saliva (Jenkins et al. 1995, Cone et al. 1997). Huestis and Cone (2004) suggested that prolonged times of detection in OF were attributed to the release of drugs from formed depot.

Furthermore, similar concentration profiles for cocaine in OF as the described in this chapter were reported by Reichardt (2014). Reichardt's study was however conducted over a period of one hour with C_{max} obtained immediately after ingestion. The T_{last} reported by Reichardt was one hour, which was in agreement with the T_{last} obtained in this study as concentrations of cocaine in OF remained positive at the EWDTS cut-off after one hour of ingestion of coca tea. Comparison between the study reported by Reichardt and the reported in this chapter showed that cocaine concentrations in OF were considerably lower in Reichardt's study to those obtained in the present study. Mean cocaine concentrations in OF of 160 ng/mL (30 minutes) and 24 ng/mL (one hour) were reported by Reichardt (2014). Mean cocaine concentrations in OF for Group A were obtained at 225 ng/mL (30 minutes) and 61 ng/mL (one hour). Variation in dose and type of tea used could have been the primary reason for the differences between the studies. However, variability between individuals and ways of drinking, i.e. speed, time spent on drinking and swirling; could have also contributed to differences in concentration as it was mentioned before.

Significant intra-individual variability was seen in both groups (Table 6.4), especially for participants P7 (Group A) and P21 (Group B), who showed extreme OF cocaine

contamination, i.e. high cocaine concentration in OF. Participants P13 and P15 (Group A) and P27 (Group B) showed low contamination, i.e. low cocaine concentration in OF. The differences in participants' profiles were primarily attributed to inter-individual variability of the oral cavity and absorption of cocaine in oral tissues. However, differences in metabolising enzymes, enhanced or reduced metabolism and genetic factor could have also influence in the inter-individual variability (Scheidweiler et al. 2010, Allen 2011). It is probable that changes in the speed of drinking or swirling of coca tea could have also contributed to the variations in release profiles, but further studies are required to confirm this. No correlation was seen between the five participants (P7, P13, P15, P21 and P27) that showed extreme or low contamination and the demographics of the participants. Three participants were female (P13, P15 and P27) and two were male (P7 and P21). None of these participants reported having medical conditions and their BMI values were within the normal range (19.5-22.7 Kg/cm^2). From the five participants only one participant was a smoker (P27). The high and low concentrations of cocaine in OF from these five participants could not be related to previous consumption of cocaine as none of them reported previous consumption of coca tea or cocaine.

6.3.4 Concentration of BZE in oral fluid following the ingestion or swirling of a cup of coca tea

BZE concentrations in OF samples before the consumption of coca tea (pre-dose samples) ranged between 1.4–2.0 ng/mL for all participants. The concentration range for the group that ingested the tea (Group A) was 1.4–1.8 ng/mL. The mean concentration range for the group that swirled the tea (Group B) was 1.5–2.0 ng/mL. Table 6.6 shows the descriptive statistics (mean, median, standard deviation, minimum and maximum values) of BZE concentration at each collection time following the ingestion or swirling

of a cup of coca tea. Summary of BZE concentrations in OF following the ingestion or swirling of a cup of coca tea is presented in Appendix E Table 2.

Table 6.6 BZE concentration in neat OF following the ingestion or swirling of a cup of coca tea.

Collection time (min)	Concentration Group A – Ingested (ng/mL) (*n* = 2)*					Concentration Group B - Swirled (ng/mL) (*n* = 2)*				
	Mean	Median	SD	Min	Max	Mean	Median	SD	Min	Max
0	1.5	1.4	0.1	1.4	1.8	1.6	1.5	0.2	1.5	2
10	118	61	161	9	603	448	348	360	5	1404
20	50	22	75	6	300	93	59	109	4	442
30	26	21	26	4	114	29	21	32	3	161
60	29	27	30	4	131	8	8	5	2	25
120	21	23	17	0	60	3	2	2	2	10
180	22	11	20	2	63	2	2	1	2	5
240	20	21	14	2	60	2	2	1	0	4

Group A: Participants ingested one cup of coca tea (data collected using one collection device at each time point). Group B: Participants swirled a cup of coca tea (data collected using two collection devices at each time point). A: OF sample collected from the right side of the mouth. B: OF sample collected from the left side of the mouth. SD: Standard deviation. *n:* Each sample was analysed in duplicate and values are presented as mean values. LOQ: 0.5 ng/mL BZE in buffered oral fluid; LOQ in neat OF was 2 ng/mL (the dilution factor from collection device was 4)

The EWDTS guidelines for BZE confirmatory testing in OF suggest a cut-off concentration of 8 ng/mL (EWDTS 2015). Based on this cut-off, none of the participants from Group A - Ingested or Group B – Swirling tested positive for BZE in the control sample (pre-dose sample). Similar results were obtained at the analytical LOQ cut-off (2 ng/mL) as all participants reported negative for the presence of BZE in OF.

Figure 6.3 illustrates the concentration profiles of BZE in OF for a period of four hours following ingestion or swirling of coca tea. Furthermore, Table 6.7 summarises the maximum BZE concentration (Cmax), times of maximum concentration (T_{max}), time of last detection ($T_{0\text{-last}}$) and area under the curve ($AUC_{0\text{-last}}$) for both groups. Mean maximum concentrations of BZE in OF (C_{max}) of 118 ng/mL (Group A) and 448 ng/mL (Group B) were observed in the first sample collected after 10 minutes following ingestion or swirling of the tea. Based on the EWDTS and LOQ cut-offs all participants

tested positive for BZE in the sample collected after 10 minutes of the ingestion or swirling of the tea.

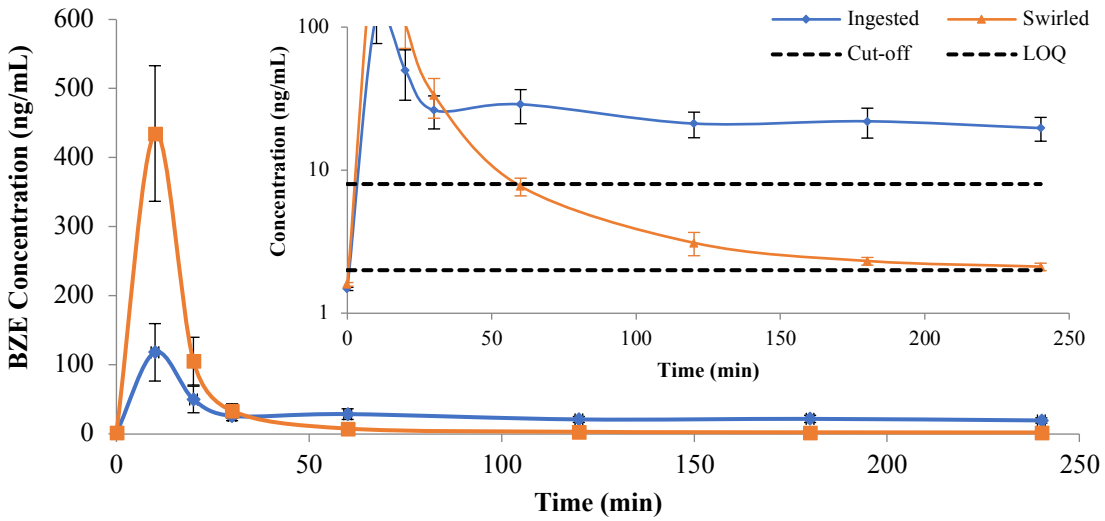


Figure 6.3 Mean concentrations of BZE in OF following the ingestion or swirling of a cup of coca tea. Bars are standard deviation. Data illustrated in full scale and with a zoom into the low concentration region. Dotted lines indicate the following cut-off concentrations: Limit of quantification (LOQ) (2 ng/mL), EWDTS (8 ng/mL).

Table 6.7 Maximum concentration, time at maximum concentration, detection window and area under the curve for BZE in OF following the ingestion (Group A) or swirling (Group B) of a cup of coca tea.

Group A – Ingested					Group B – Swirled				
Participant	**C_{max} (ng/mL)**	**T_{max} (min)**	**T_{last} (min)**	**$AUC_{0\text{-}last}$ (ng/mL/min)**	**Participant**	**C_{max} (ng/mL)**	**T_{max} (min)**	**T_{last} (min)**	**$AUC_{0\text{-}last}$ (ng/mL/min)**
P1	174	10	>240	154	**P16**	539	10	120	7476
P2	62	20	>240	84	**P17**	615	10	120	5586
P3	69	10	>240	123	**P18**	199	10	60	4447
P4	34	10	>240	29	**P19**	348	20	120	6198
P5	110	180	>240	177	**P20**	89	10	60	1723
P6	25	10	>240	80	**P21**	1289	10	120	26699
P7	603	10	>240	362	**P22**	816	10	180	11629
P8	58	10	>240	103	**P23**	328	10	60	3794
P9	55	10	>240	127	**P24**	212	10	120	3609
P10	363	10	>240	158	**P25**	1008	10	60	7662
P11	31	10	>240	63	**P26**	146	10	120	1683
P12	64	10	>240	45	**P27**	5	10	-	752
P13	9	10	>240	15	**P28**	487	10	60	7725
P14	116	10	>240	63	**P29**	489	10	60	7199
P15	16	10	>240	15	**P30**	223	10	120	4518
Mean	119	18	-	107		453	11	99	6713
Median	62	10	-	84		348	10	120	5586
SD	160	28	-	87		359	3	38	6220
Min	9	10	>240	15		5	10	60	752
Max	603	120	>240	362		1289	20	180	26699

C_{max}: Maximum concentration; T_{max}: Time at maximum concentration; T_{last}: Time of last detection at EWDTS cut-off (8 ng/mL); $AUC_{0\text{-}last}$: Area under the curve. SD: standard deviation.

Similarly to what was observed for cocaine, a rapid decrease in concentration between both groups was seen over the first thirty minutes. The mean concentrations of BZE in OF were 26 and 29 ng/mL for Group A (Ingested) and Group B (Swirling) respectively. After one hour, 56% (Mean 8.0 ng/mL; Median 7.8 ng/mL) of the participants from Group B reported positive at both cut-offs. The time of last detection at the EWDTS cut-off for Group B was two hours (Mean: 99 ng/mL; Median: 120 ng/mL). Only one participant (P22) reported positive in Group B after two hours of having swirled the tea but his concentration in OF decreased below the cut-off after three hours. The time of last detection at the assay's LOQ was longer for Group B as 60% of the population remained positive after four hours. The time of last detection at the EWDTS and LOQ cut-offs for Group A could not be determined as all participants had detectable concentrations of BZE in OF after four hours (Table 6.7).

Higher concentrations of BZE in OF were obtained during the first hour after coca tea was swirled (Group B) than when it was ingested (Group A) (Figure 6.3), as was observed for the analysis of cocaine in OF. A Mann- Whitney (U) test indicated that there were significant differences between the C_{max} ($U = 36$, $p = 0.002$) and $AUC_{0\text{-last}}$ ($U = 0$, $p < 0.001$) between Group A - Ingestion and Group B - Swiling. Samples that tested positive at the EWDTS cut-off collected at 20 and 30 minutes showed no significant differences between the groups (20 minutes: $U = 138$, $p = 0.04$; 30 minutes: $U = 221$, $p = 0.92$). After one hour of ingestion/swirling, significant differences were seen between the groups ($p < 0.001$).

The amount of BZE in OF during the initial 30 minutes post ingestion/swirling of the tea was less than 20% the concentration observed for cocaine. This result was in agreement with the amount of analytes present in a cup of coca tea as the percentage of

BZE in respect to cocaine was < 20% (BZE: 0.8 ± 0.04 mg; COC: 6.7 ± 0.04 mg). The fact that BZE was present in the coca tea indicated that detection of BZE immediately post-consumption and up to a period of 30 minutes could have reflected the concentration of BZE in the coca tea (because of oral contamination, i.e. remaining coca tea in the oral cavity) and not its concentration in OF resulting from the excretion of BZE from drug depots or systemic circulation.

After 35 minutes of dosage (ingestion/swirling) the concentration of BZE in OF in the group that ingested the tea (Group A) was greater than the group that swirled the tea (Group B), as observed for cocaine. This difference in concentration was also seen in the T_{last} as BZE was detected at concentrations higher than the EWDTS cut-off during the length of the study (four hours). The increase in BZE concentration in Group A could have been the result of the metabolism of cocaine in the body, which was subsequently eliminated from the systemic circulation into the OF. Although, BZE could have also been released from drug depots formed in the oral cavity.

As per cocaine, similar BZE concentration profiles in OF have been reported following oral administration (smoke doses of 40 and 42 mg or intranasal dose of 32 mg) (Jenkins et al. 1995, Cone et al. 1997). Jenkins et al. (1995) reported C_{max} immediately after dosage (2-5 minutes). Cone et al. (1997) reported C_{max} for BZE in OF slightly delayed with T_{max} ranging between 0.3-3 hours following intranasal dose of 32 mg and 0-2 hours following smoking of 40 mg. Furthermore, the authors reported that the concentration of BZE in OF was cleared (below the EWDTS cut-off) after 4-12 hours of dosage. Hence agreeing with the time of last detection obtained for Group A – Ingestion.

The results for BZE were also in agreement with the results reported by Reichardt (2014) as C_{max} (Mean: 174 ng/mL BZE in OF) was obtained immediately after ingestion of a cup of coca tea. Reichardt reported a rapid decrease in BZE concentration within 15 minutes of dosage to a mean concentration of 17 ng/mL that did not change over 45 minutes. These results were also in agreement with the results observed in this study. The differences in C_{max} and T_{last} obtained in this study and those reported by Reichardt were attributed to differences in the dosage as mentioned in the previous section (Section 6.3.3).

BZE to cocaine ratios (BZE/COC) generally increased with time for both groups as indicated in Figure 6.4. The slight increase in BZE/COC ratio seen for Group B - Swirling could be attributed to differences in the rate at which BZE and cocaine were released from drug depots into OF as BZE could have been released more rapidly from drug depots than cocaine thus increasing the BZE/COC ratio. BZE is more hydrophilic than cocaine and therefore could be less likely to bond to lipids present in the oral tissues such as the connective tissue.

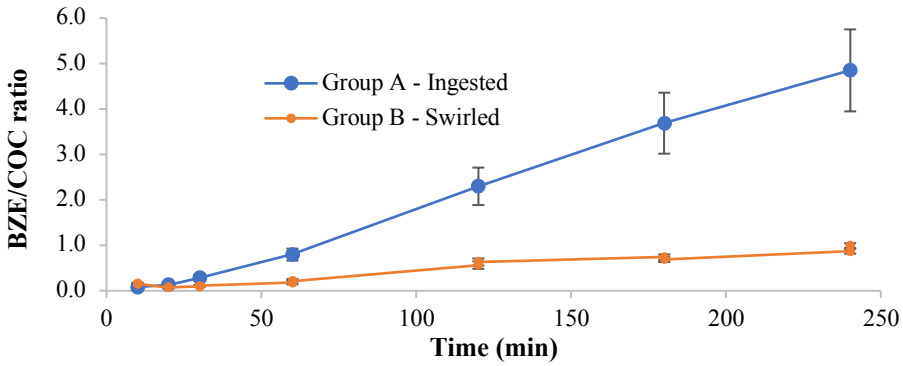


Figure 6.4 Mean BZE/COC concentration ratio over a period of four hours following the ingestion (Group A) or swirling (Group B) of a cup of coca tea.

BZE/COC ratio could have increased as a result of cocaine degradation. The results of stability studies reported in Chapter 5 indicated that the BZE concentration in BOF

increased up to 27% of its initial concentration because of cocaine degradation when samples were stored at room temperature over a period of eight days. Storage of OF samples could not be controlled over the time the samples were shipped from Colombia to the UK (seven days) hence the concentration of BZE in OF samples could have increased from its initial concentration during the shipping period. The significant increase of BZE/COC ratio obtained by Group A denoted that an increased amount of BZE or a decreased amount of cocaine was being released into OF over time when the tea was ingested rather than swirled. BZE has been reported to have wider window of detection and longer half-life in OF than cocaine, which indicates that cocaine is eliminated faster than its metabolite (Scheidweiler et al. 2010). Furthermore, it has been reported that concentrations of BZE were higher in plasma than in OF and that its concentration increased with time because of cocaine degradation. Thus, it is possible to suggest that the increased BZE/COC ratio in OF was the result of the metabolism of cocaine which increased the BZE blood concentration and consequently elevated the concentration of BZE in OF over time.

Significant intra-individual variability was seen in both groups (Table 6.6) especially for participants P7 and P21 who showed extreme OF BZE contamination (high concentrations of BZE in OF) and participants P13 and P27 who showed low contamination (low concentrations of BZE in OF). After excluding the extreme values, the mean and median values for C_{max} and $AUC_{0\text{-}last}$ did not change significantly and significant differences were still obtained between the groups. The C_{max} values for Group A were Mean: 85 ng/mL and Median: 60 ng/mL, for Group B were Mean: 423 ng/mL and 386 ng/mL respectively. The $AUC_{0\text{-}last}$ values for Group A were Mean: 88 ng/mL and Median: 82 ng/mL, for Group B were Mean: 5286 ng/mL and 5052 ng/mL respectively. The differences in concentration between groups were also attributed to the inter-

individual variability of the oral cavity, absorption of cocaine in oral tissues and the differences in drinking or swirling of the tea as discussed previously.

6.3.5 Concentration of AEME in oral fluid following the ingestion or swirling of a cup of coca tea

AEME was not detected in any of the pre-dose OF samples from participants that ingested coca tea (Group A). Only one participant (P29) that swirled the tea (Group B) reported concentrations above the LOQ cut-off (2 ng/mL). Summary of AEME concentrations in OF following the ingestion or swirling of a cup of coca tea is presented in Table 6.8.

Table 6.8 AEME concentration in neat OF following the ingestion or swirling of a cup of coca tea.

Collection time (min)	Concentration Group A – Ingested (ng/mL) (n = 2)*					Concentration Group B - Swirled (ng/mL) (n = 2)*				
	Mean	Median	SD	Min	Max	Mean	Median	SD	Min	Max
0	0	0	0	0	0	0	0	0	0	1
10	207	167	161	37	655	542	504	291	121	1275
20	92	58	105	12	431	262	220	160	20	665
30	43	22	51	3	193	139	110	113	0	581
60	6	1	12	0	46	23	15	27	0	119
120	0	0	0	0	1	1	0	3	0	12
180	0	0	0	0	0	0	0	1	0	3
240	0	0	0	0	0	0	0	0	0	0

Group A: Participants ingested one cup of coca tea (data collected using one collection device at each time point). Group B: Participants swirled a cup of coca tea (data collected using two collection devices at each time point). A: OF sample collected from the right side of the mouth. B: OF sample collected from the left side of the mouth. SD: Standard deviation. *n:* Each sample was analysed in duplicate and values are presented as mean values. LOQ: 0.5 ng/mL cocaine in buffered oral fluid; LOQ in neat OF was 2 ng/mL (the dilution factor from collection device was 4)

In this study all participants reported positive for AEME at the LOQ cut-off of 2 ng/mL immediately after the ingestion or swirling of a cup of coca tea. Figure 6.5 illustrates the concentration profile of AEME in OF for a period of four hours following ingestion or swirling of coca tea. Furthermore, Table 6.9 summarises of the maximum AEME concentration (C_{max}), times of maximum concentration (T_{max}), time of last detection (T_{0}-

last) and area under the curve ($AUC_{0\text{-last}}$) for both groups. Mean maximum concentrations of AEME in OF (C_{max}) of 207 ng/mL (Group A) and 542 ng/mL (Group B) were observed in the first sample collected after 10 minutes following ingestion or swirling of the tea.

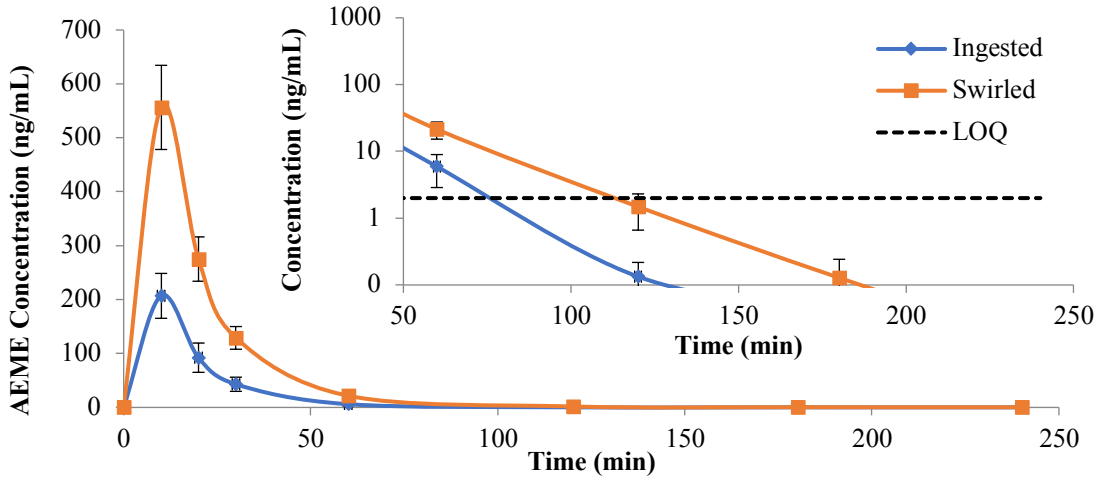


Figure 6.5 Mean concentrations of AEME in OF following the ingestion or swirling of a cup of coca tea. Bars are standard deviation. Dotted lines indicate the following cut-off concentrations: Limit of quantification (LOQ) (2 ng/mL).

Table 6.9 Maximum concentration, time at maximum concentration, detection window and area under the curve for AEME in OF following the ingestion (Group A) or swirling (Group B) of a cup of coca tea.

Group A – Ingested					Group B - Swirled				
Participant	**C_{max} (ng/mL)**	**T_{max} (min)**	**T_{last} (min)**	**$AUC_{0\text{-last}}$ (ng/mL/min)**	**Participant**	**C_{max} (ng/mL)**	**T_{max} (min)**	**T_{last} (min)**	**$AUC_{0\text{-last}}$ (ng/mL/min)**
P1	167	10	60	49	**P16**	466	10	60	8979
P2	175	20	60	49	**P17**	433	10	60	5982
P3	165	10	30	36	**P18**	544	10	120	15072
P4	367	10	60	120	**P19**	528	20	60	8982
P5	347	180	60	107	**P20**	125	10	60	1867
P6	67	10	30	16	**P21**	1240	10	120	42708
P7	655	10	60	245	**P22**	491	10	120	13226
P8	253	10	30	56	**P23**	570	10	60	9860
P9	141	10	60	38	**P24**	395	10	60	8670
P10	275	10	30	51	**P25**	919	10	60	18111
P11	37	10	30	10	**P26**	237	10	60	2495
P12	132	10	30	31	**P27**	209	10	60	9048
P13	58	10	30	11	**P28**	851	10	60	25478
P14	221	10	60	75	**P29**	518	10	60	11724
P15	54	10	30	22	**P30**	596	10	60	12517
Mean	208	18	44	61		542	11	72	13149
Median	167	10	30	49		518	10	60	10679
SD	161	28	15	60		286	3	25	8715
Min	37	10	30	10		125	10	60	2666
Max	655	120	60	245		1240	20	120	35595

C_{max}: Concentration at maximum concentration; T_{max}: Time at maximum concentration; T_{last}: Time of last detection at LOQ (2 ng/mL); $AUC_{0\text{-last}}$: Area under the curve. SD: standard deviation.

Like it was observed for BZE, a rapid decrease in AEME concentration in both groups was seen over the first thirty minutes to a mean concentration of 43 and 139 ng/mL (Group A- Ingested and Group B - Swirling respectively). Mean concentration values at one hour were 5.9 ng/mL for Group A and 23.4 ng/mL for Group B. Mean values for T_{last} at the assay's LOQ for Group A was 44 minutes and for Group B was 72 minutes (Table 6.9). Three participants (P18, P21 and P22) from Group B tested positive at the assay's LOQ two hours post dosage, but the concentration decreased below the LOQ cut-off three hours post dosage.

Figure 6.5 illustrates that higher concentrations of AEME in OF were obtained during the first hour post dosage when the coca tea was swirled than when it was ingested as was obtained for the analysis of cocaine and BZE. A Mann-Whitney (U) test indicated that there were significant differences between the C_{max} ($U = 31$, $p < 0.001$) and $AUC_{0\text{-}last}$ ($U = 0$, $p < 0.001$) in the two groups. There were significant differences in AEME concentrations between the two groups at 20 minutes ($U = 0.67$, $p < 0.001$), 30 minutes ($U = 0.63$, $p < 0.001$) and 60 minutes ($U = 0.2$, $p = 0.001$). After two hours there were no significant differences between the groups ($p > 0.05$).

Contrary to what was observed for cocaine and BZE, the concentrations of AEME in OF in the Group B (Swirling) remained below Group A (Ingested) at all times. This was also seen in the T_{last} as participants that swirled the tea had longer times (1-2 hours) than the participants that ingested the tea (30 minutes). The fact that higher amounts of cocaine BZE and also AEME were detected in the participants during the first hour after swirled the tea, strongly suggest that the time spent by the participants swirling the tea increased the amount of analyte being absorbed in the oral cavity even when both groups of participants had the same dosage. AEME is further metabolised into anhydroecgonine

(Kintz et al. 1997, Fandiño et al. 2002), hence it is possible that participants that ingested the tea could have metabolised AEME into anhydroecgonine and this was eliminated in the OF. However, this result could not be confirmed as anhydroecgonine was not monitored in this study.

The amount of AEME in OF during the first 30 minutes post ingestion/swirling of the tea was less than 30% the concentration observed for cocaine. This result was in agreement with the amount of analyte present in a cup of coca tea as the percentage of AEME in respect to cocaine was 17% (AEME: 1.2 ± 0.03 mg; COC: 6.7 ± 0.04 mg). Since AEME was present in the coca tea, it can be suggested that detection of AEME could be reflecting the concentration of AEME in the coca tea as a result of oral contamination as well as its release from drug depots into OF.

The concentration profiles of AEME in OF were in agreement to the profiles reported by Jenkins et al. (1995) and Cone at al. (1997) who reported AEME concentrations following oral administration of cocaine base (smoke doses of 40 and 42 mg respectively). These authors reported that AEME was detected in OF at high concentrations with C_{max} ranging 558-4374 ng/mL (Jenkins et al. 1995) and 51-303 ng/mL (Cone et al. 1997), within two minutes of smoking. Furthermore, it was reported that AEME rapidly decreased with time over a period of 15-30 minutes, which was similar to the results presented in this study (30-60 minutes). From the two studies that were reported, it was noticed that although the participants smoked similar dosages, there were significant differences in AEME concentration in OF (e.g. C_{max}) which were cleared within the same period of time. These results could indicate that there was a rapid dissolution of AEME in the OF which allowed an effective clearance by swallowing within 15-30 minutes of drug administration. Based on these results it can be suggested

that after 30 minutes of ingestion/swirling of a cup of coca tea, the detection of AEME in OF could have been the result of its release from drug depots. The shorter T_{last} for AEME (30 min for Group A and 60 minutes for Group B) compared with the T_{last} for cocaine (three hours for Group A and two hours for Group B) could also suggest that AEME diffused more rapidly across oral membranes, which could be explained by the lipophilic nature of AEME and its potential bonding to lipids present in the oral tissues such as the connective tissue.

The results presented in this Chapter were different from the results reported by Reichardt (2014), who reported a random distribution of AEME amongst OF samples following ingestion of a cup of coca tea. In Reichardt's study, nine out of twenty volunteers tested positive in at least one OF sample during the one-hour collection period. AEME concentrations in OF in Reichardt's study ranged between 1-322 ng/mL with C_{max} observed immediately after ingestion. Re-analysed the OF samples one year after sample collection, showed similar results as the initial reported. Reichardt reported that positive samples could have been obtained from AEME formation in OF following the consumption of coca tea and the possible instability of the samples by thermal decomposition during storage or during the tea preparation.

It is unlikely that AEME could have been detected in OF as a result of its formation after the collection of the OF samples (chemical reaction during storage or during analytical analysis) as mentioned by previous authors (Reichardt 2014). This statement can be confirmed by the results presented in Section 6.3.1, which confirmed that AEME was present in the coca tea and the plant material (*E. novogranatense* var. *nov*). This AEME intrinsic to the coca leaves could have subsequently be absorbed into oral tissues and released into OF. Since clear AEME profiles were obtained for all the participants

that ingested or swirled a cup of coca tea, it can be suggested that the random distribution observed by Reichardt could have been the result of poor storage conditions.

6.3.6 Concentration of EME in oral fluid following the ingestion or swirling of a cup of coca tea

All pre-dose OF samples tested negative for EME from participants than ingested or swirled coca tea. Two participants (P21 and P29) that swirled the tea (Group B) showed low concentrations of EME but these were below the LOQ cut-off (4 ng/mL). Summary of EME concentrations in OF following the ingestion or swirling of a cup of coca tea is presented in Table 6.10.

Table 6.10 EME concentration in neat OF following the ingestion or swirling of a cup of coca tea.

Collection time (min)	Concentration Group A – Ingested (ng/mL) (*n* = 2)*					Concentration Group B – Swirled (ng/mL) (*n* = 2)*				
	Mean	**Median**	**SD**	**Min**	**Max**	**Mean**	**Median**	**SD**	**Min**	**Max**
0	**0**	0	0	0	0	**0**	0	1	0	2
10	**1723**	161	2725	0	8911	**12178**	9954	9401	196	31823
20	**807**	45	1277	0	4181	**4594**	3056	4736	60	16561
30	**389**	33	822	0	3194	**2902**	2502	2474	52	10308
60	**66**	6	126	0	450	**653**	418	795	0	3239
120	**11**	0	22	0	76	**88**	59	86	0	240
180	**4**	0	15	0	58	**32**	22	33	0	123
240	**1**	0	3	0	11	**15**	2	21	0	77

Group A: Participants ingested one cup of coca tea (data collected using one collection device at each time point). Group B: Participants swirled a cup of coca tea (data collected using two collection devices at each time point). A: OF sample collected from the right side of the mouth. B: OF sample collected from the left side of the mouth. SD: Standard deviation. *n:* Each sample was analysed in duplicate and values are presented as mean values. LOQ: 1.0 ng/mL cocaine in buffered oral fluid; LOQ in neat OF was 4 ng/mL (the dilution factor from collection device was 4)

All participants reported positive for EME at the LOQ cut-off of 4 ng/mL in the first sample collected after the ingestion or swirling of a cup of coca tea, except the participant P6 (Group A) who reported negative for EME in all OF samples. Like for AEME, there

are no proposed EWDTS guidelines for EME confirmatory testing in OF. Therefore, the results were presented based on the LOQ cut-off value (4 ng/mL).

Figure 6.6 illustrates the concentration profile of EME in OF for a period of four hours following ingestion or swirling of coca tea. Furthermore, Table 6.11 summarises of the maximum EME concentration (C_{max}), times of maximum concentration (T_{max}), time of last detection ($T_{0\text{-last}}$) and area under the curve ($AUC_{0\text{-last}}$) for both groups. EME was the analyte that presented the highest concentration after ingestion/swirling of coca tea. In general, the concentration of EME was expected to be higher than cocaine as EME was present in a cup of coca tea in a higher percentage (13%) than cocaine (COC: 6.7 ± 0.04 mg; EME: 87.2 ± 5.02 mg). Mean maximum concentrations of EME in OF (C_{max}) of 1723 ng/mL (Group A) and 12178 ng/mL (Group B) were observed in the first sample collected after 10 minutes following ingestion or swirling of the tea, as per cocaine, BZE and AEME.

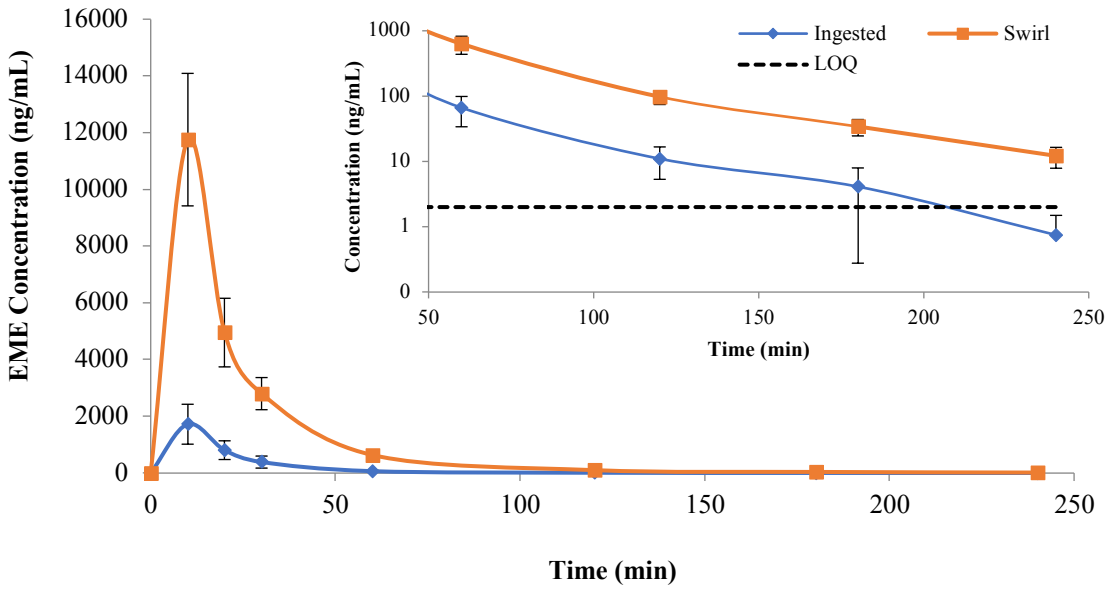


Figure 6.6 Mean concentrations of EME in OF following the ingestion or swirling of a cup of coca tea. Bars are standard deviation. Dotted line indicates the limit of quantification (LOQ) (4 ng/mL).

Table 6.11 Maximum concentration, time at maximum concentration, detection window and area under the curve for EME in OF following the ingestion (Group A) or swirling (Group B) of a cup of coca tea.

Group A – Ingested					Group B – Swirled				
Participant	**C_{max} (ng/mL)**	**T_{max} (min)**	**T_{last} (min)**	**$AUC_{0\text{-}last}$ (ng/mL/min)**	**Participant**	**C_{max} (ng/mL)**	**T_{max} (min)**	**T_{last} (min)**	**$AUC_{0\text{-}last}$ (ng/mL/min)**
P1	42	10	10	4	**P16**	29244	10	>240	894149
P2	56	10	60	9	**P17**	20105	10	180	199898
P3	118	10	10	10	**P18**	7514	10	>240	328580
P4	161	10	30	38	**P19**	9483	10	180	162613
P5	160	10	120	35	**P20**	2903	10	180	131818
P6	0	0	0	0	**P21**	28939	10	180	573879
P7	382	10	30	100	**P22**	13831	10	>240	233468
P8	150	10	30	23	**P23**	14524	10	>240	320898
P9	65	10	60	11	**P24**	6723	10	180	43242
P10	8911	10	120	2207	**P25**	15481	10	>240	158808
P11	1354	10	120	519	**P26**	1812	10	60	33549
P12	4101	10	120	1094	**P27**	5009	10	120	718910
P13	1802	10	60	389	**P28**	19866	10	>240	597038
P14	6560	10	120	2844	**P29**	5179	10	60	165776
P15	2352	10	240	1127	**P30**	4394	10	180	259930
Mean	1748	10	75	561		12334	11	184	321504
Median	161	10	60	38		9483	10	180	233468
SD	2730	0	64	892		8969	0	62	256924
Min	0	10	0	0		1812	10	60	33549
Max	8911	10	240	2844		29244	10	240	894149

C_{max}: Concentration at maximum concentration; T_{max}: Time at maximum concentration; T_{last}: Time of last detection at LOQ (4 ng/mL); $AUC_{0\text{-}last}$: Area under the curve. SD: standard deviation.

A rapid decrease in concentration for both groups was observed over the first hour to a mean concentration of 66 and 653 ng/mL for Group A and B respectively. After two hours, 27% (Group A - Ingested) and 77% (Group B - Swirling) of the population still tested positive at the LOQ cut-off. Mean concentration values at two hours were 11 ng/mL for Group A and 88 ng/mL for Group B. The time of last detection at the LOQ for Group A was one hour and for Group B was three hours (Table 6.11). One participant (P15) from the Group A (Ingested) and six participants (P16, P18, P22, P23, P25 and P28) from Group B (Swirling) had detectable concentrations of EME in OF after four hours. The mean concentration of EME present in the last collected sample (collection time of 240 minutes) was 15 ng/mL for Group B (Figure 6.6).

Similarly to cocaine, BZE and AEME, higher concentrations of EME in OF samples collected during the first hour were obtained when the coca tea was swirled than when it was ingested (Figure 6.6). A Mann-Whitney (U) test indicated that there were significant differences between the C_{max} ($U = 0$, $p < 0.001$) and $AUC_{0\text{-last}}$ ($U = 0$, $p < 0.001$) in the two groups. Equally to the C_{max}, there were significant differences between EME concentrations between the two groups at all times of collection ($p < 0.001$). EME concentrations in OF in the Group A (Ingested) remained below than the Group B (Swirling) at all times as was observed for AEME. This was also reflected in the T_{last} as participants that had swirled the tea exhibited longer times (three hours) than the participants that ingested the tea (one hour). The elevated concentrations of EME in OF in both groups were primarily attributed to the release of EME from drug depots. High concentration of EME in OF were expected as the amount of EME in coca leaves were considerable high compared with other constituents like cocaine (87 mg EME per one gram of coca leaves). These EME could had formed drug depots in the oral cavity and subsequently increase the concentration of EME in OF. It is possible that for Group A – Ingested, the concentration of EME in OF could have been the result of the elimination of EME from drug depots as well as from the systemic circulation because EME can be produced from the metabolism of cocaine.

Considerable differences were seen between the results obtained for EME in this study and the EME concentrations reported in OF by other authors following smoking of 40-42 mg cocaine base (Jenkins et al. 1995, Cone et al. 1997) and drinking 3 mg of cocaine from mate de coca (Strano-Rossi et al. 2008). These authors reported that EME was detected at similar concentrations for BZE in OF and at consistently low concentrations compared to cocaine. The mean C_{max} for EME in OF reported by these authors following smoking cocaine base (Jenkins: 50 ng/mL; Cone:34 ng/mL; Strano-Rosi: 23 ng/mL) were

up to 40 times lower than those reported in this study (Group A – Ingested: 1723 ng/mL; Group B – Swirling 12178 ng/mL). Jenkins and Cone suggested that EME was detected as a result of the metabolism of cocaine because C_{max} was obtained at later times (T_{max} ranged between 20-120 minutes) than the samples collected immediately after drug administration (two minutes).

Contrary to the results reported following the smoking of cocaine base, Reichardt (2014) reported elevated oral contamination following the ingestion of coca tea. Mean EME C_{max} was reported at 3239 ng/mL (24-16685 ng/mL) in OF immediately after ingestion of the tea. Reichardt reported a rapid decreased in EME concentration within 15 minutes of dosage to a mean concentration of 257 (39-1209 ng/mL) ng/mL that did not change over 45 minutes. The results presented in this chapter confirmed the results reported by Reichardt as EME were found at high concentrations in OF samples and samples tested positive at the LOQ cut-off over a period of one-hour postdose. The differences in C_{max}, T_{max} and T_{last} between the studies were attributed to differences in the dose as mentioned in the previous section (Section 6.3.3) and the study design.

The EME to cocaine ratios (EME/COC) for Group A initially increased over time from 1.4 to 1.7 in the first 30 minutes post-dose and then decreased to 0.1 at four hours. Differently, EME/COC ratios for Group B increased from 4.8 to 9.4 during the first three hours. The differences between the EME/COC ratios could indicate that a greater amount of EME depots were formed in the oral tissue when the tea was swirled and subsequently released into OF.

6.3.7 Concentration of cocaethylene (CE) and norcocaine (NC) in OF following ingestion or swirling of a cup of coca tea.

The analysis of cocaine and cocaine derivatives in collected OF samples following the ingestion or swirling of coca tea showed that only AEME, BZE, COC and EME were detected in the OF samples as described above. The analytes NC and CE were not detected in any of the OF samples collected from the study participants. The absence of CE in OF samples confirmed that none of the participants consumed alcohol in the three days prior to the commencement of the study (Laizure et al. 2003, Lewis et al. 2004). The absence of NC in the samples indicated that cocaine was not enzymatically metabolised by the liver to NC (Poon et al. 2014) or that the concentration of NC in OF was below the LOD. Neither CE and NC were detected in the coca tea.

6.3.8 Comparison of oral fluid samples collected from different sides of the mouth

OF samples were collected from different sides (left and right) of the mouth using the same collection procedure to determine whether there are differences in the concentration of analytes when two samples are collected simultaneously. The European guidelines for workplace drug testing in OF (EWDTS 2015) suggests the collection of an A and B sample which can be generated by the division of one sample from a single collection procedure, or by the collection of two separate samples. It is known that some OF collection devices cannot collect and generate two separate samples from one single sample, because of the limited volume of OF collected. When two samples are to be collected, the first aliquot must be labelled A and the second B (EWDTS 2015). In this case, the two samples should be simultaneously collected; otherwise, the samples might

not be identical. If samples are not collected simultaneously exact times of the generation of the sample must be recorded to be able to correlate the results.

Although OF samples are collected following standard collection procedures such as those described by the European guidelines for workplace drug testing in OF (EWDTS 2015), there is a lack of evidence on the equivalence of the samples (A and B) when they have not been generated from a single collection procedure.

Mean concentrations ratios for AEME, BZE, COC and EME ranged between 1.01 – 1.15 indicating that samples collected on the right (A) side of the mouth were equivalent to the left (B) side of the mouth (Table 4.12). A Mann-Whitney (U) test confirmed that there were no significant differences in concentrations obtained using the two collection devices simultaneously. The results for each analyte (A= 240 samples; B = 120 samples) were as follow: AEME (U = 6279, p = 0.077), BZE (U = 6485, p = 0.182), COC (U = 7043, p = 0.771) and EME (U = 6954, p = 0.646).

Table 6.12 Concentration ratios for samples collected on the right (A) and left (B) side of the mouth

Analyte	Concentration ratio A/B					
	Mean	**Median**	**SD**	**RSD**	**Min**	**Max**
AEME (n = 62)	1.01	0.91	0.32	29.5	0.56	2.25
BZE (n = 104)	1.15	1.00	0.63	54.6	0.32	5.63
COC (n = 119)	1.11	1.00	0.46	41.2	0.23	3.34
EME (n = 70)	1.04	1.04	0.45	43.2	0.10	2.53

A: Samples collected on the right side of the mouth; B: Samples collected on the left side of the mouth; AEME: Anhydroecgonine methyl ester; BZE: Benzoylecgonine; COC: Cocaine; EME: Ecgonine methyl ester; n: Number of samples (A/B)

Although a general comparison between the concentrations obtained in the right (A) and left (B) side of the mouth did not show any significant differences, when the ratio A/B

was calculated at each time point (paired samples) high percentages of relative standard variation (RSD) of up to 55% (for all analytes) were obtained. This variation can be observed in Figure 6.7 which illustrates the concentration ratio A/B for each participant (including all seven samples collected per participant) at the corresponding analyte. Figure 6.7 shows that most of the samples were distributed within one standard deviation (1SD) of the mean but some were within two standard deviation (2SD) or above. For AEME, 82% of the samples were within 1SD and 98% within 2SD. For BZE, 85% of the samples were within 1SD and 98% within 2SD. For cocaine, 79% of the samples were within 1SD and 96% within 2SD. For EME, 76% of the samples were within 1SD and 93% within 2SD.

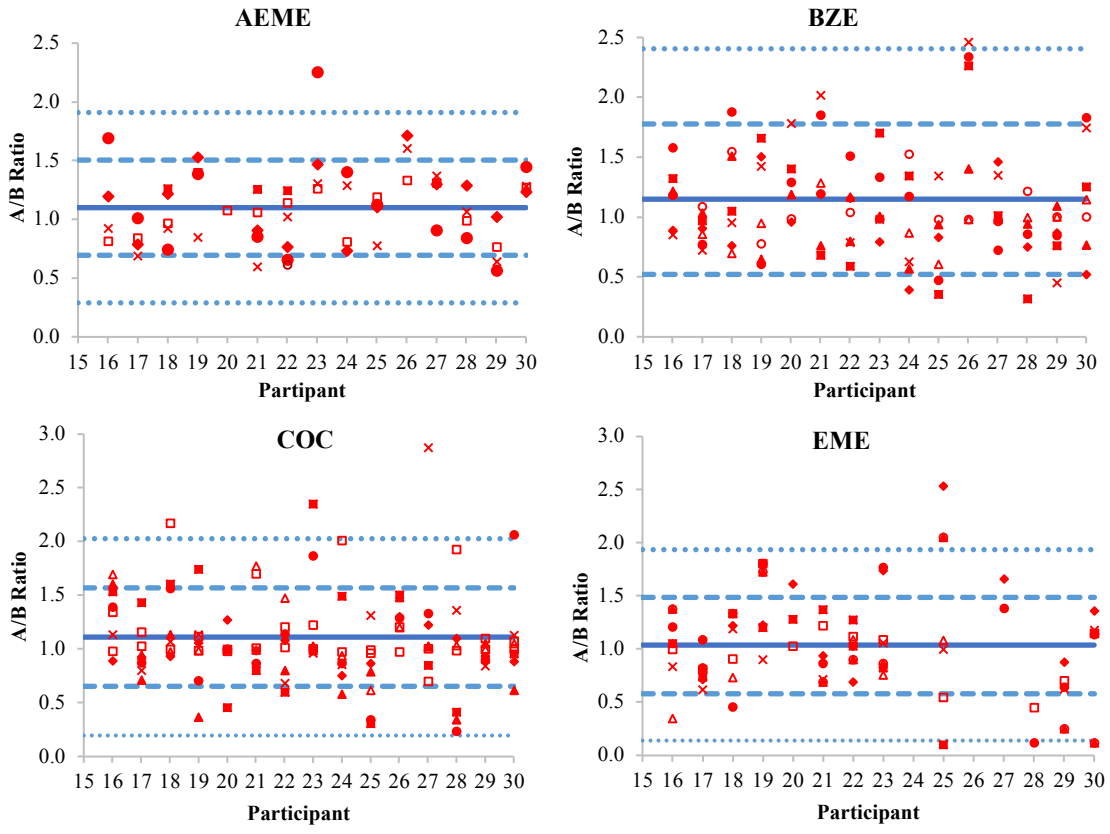


Figure 6.7 Concentration ratio for samples collected on the right (A) and left (B) side of the mouth. Red coloured marks correspond to samples collected from each participant over four-hour period following the swirling of a cup of coca tea. The different shapes indicate the different collection times for each participant. The straight line is the mean value. Dotted lines indicate one and two standard deviations.

Different factors could have contributed to the variability in A/B ratios including (1) the effect of drug depots such as the amount of drug being deposited in the oral cavity and the heterogenic formation of these depots among the tissues. (2) The rate at which the drug was released from drug depots into OF. (3) Changes in salivation (salivary flow rate) could have contributed to an increase or decrease in the dissolution of the drug and its transport from oral tissues into OF. Individual salivation can vary from 0.3 to 0.7 mL/min (Yoshizawa et al. 2013). (4) Swabbing from the surface of the oral cavity could have contributed to an increase on analyte adsorbed on the pad and consequently its concentration in OF. Although variances in the volume of OF collected using the collection swab could have contributed to the differences in concentrations of cocaine and its metabolites, this was unlikely to occur because gravimetric correction was conducted for all samples.

The comparison between concentrations obtained from the two sides of the mouth (left and right) using two devices (simultaneous collection) showed significant differences for all analytes ($p > 0.05$) except cocaine ($U = 0.145$, $p = 0.075$). However, these results were not conclusive because the concentrations of cocaine and metabolites following the ingestion of a cup of coca tea collected using one device were significantly different from the concentrations obtained when the tea was swirled and two devices were used (Section 6.4 to Section 6.7). Further studies need to be conducted in order to confirm the equivalence of results when samples have been collected with one or two collection devices as significant differences might be obtained in individual cases. Differences in the concentration of analytes from two samples collected simultaneously could have an impact on OF drug testing.

6.4 CONCLUSIONS

The initial results presented in this chapter revealed that 1.2 ± 0.2 mg AEME, 0.8 ± 0.2 mg BZE, 6.7 ± 1.0 mg COC and 87.2 ± 25.1 mg EME were present in a cup of Colombian coca tea prepared with tea bags from the Nasa Community (*E. novogranatense* var. *nov*.). Quantitative analysis of a methanolic extract of coca leaves confirmed that AEME was present in the plant material. This result could have a great impact on drug testing as AEME could be detected in OF samples following consumption of coca leaves or coca tea and therefore wrongly indicate (giving false positives) previous use of crack-cocaine.

The results obtained from the questionnaire filled by the participants of the study (Undergraduate students from the National University of Colombia) confirmed that consumption of coca leaves is not considered a psychoactive substance and an addiction in regions where it is customarily used as it was previously mentioned by Rubio et al. (2014). The results of a questionnaire indicated that coca tea consumption was related to traditional reasons, altitude sickness, test or flavour, interest, curiosity, to increase energy or elevate mood.

Positive detection of cocaine, BZE, AEME and EME at the EWDTS (8 ng/mL for cocaine and BZE) and LOQ (2-4 ng/mL for all analytes) was obtained following the ingestion or swirling of a cup of Colombian coca tea. The concentration profile of all analytes in OF was similar for all analytes in both groups. Although, participants that swirled coca tea (Group B) showed significantly higher concentrations (C_{max} and $AUC_{0\text{-}last}$) in comparison with the group that ingested the tea (Group A) during the first hour after dosage.

The time of last detection (median values) for the participants that ingested a cup of coca tea (Group A) was 30 minutes (AEME), three hours (cocaine) and one hour (EME). The concentration of BZE in OF for Group A (Ingested) remained positive during the length of the study. For Group B(Swirling), the time of last detection (median values) was 60 minutes for AEME, two hours for cocaine and BZE, and three hours for EME. Comparison between both groups showed that time of last detection for cocaine and BZE was higher for the drinkers (Group A) than the swirlers (Group B). An increase in cocaine and BZE concentration in OF from the participants that ingested the tea (Group A) after 75 minutes (COC) and 35 minutes (BZE) of ingestion indicated that these analytes were released not only from drug depots but from the systemic circulation. Comparison between the values obtained for time of last detection for all analytes indicated that the analytes could have been released from drug depots into OF at different rates.

The results of the study suggested that differences in concentration profile and time of last detection for all analytes could have been the result of differences in the amount of drug depots formed in the oral cavity and its subsequent release into OF. It is possible that a greater amount of drug depots was formed in the oral cavity when the tea was swirled than when it was ingested as a result of increased time of exposure to the tea.

This study supported the results reported by Reichardt (2014) for the positive detection of cocaine and BZE but demonstrated that AEME was detected in OF samples following the ingestion/swirling of coca tea. In this study no random distribution of AEME and CE were observed. CE and NC were not detected in any of the collected OF samples.

The analysis of paired OF samples collected simultaneously from different sides of the mouth showed that there were no significant differences between the mean concentration

of cocaine and metabolites from OF samples collected on the right (A) and left (B) side of the mouth. Analysis of the concentration ratios (A/B) from paired samples across the collected samples showed that most concentration ratios (76-85%) were distributed within one standard deviation of the mean. Although no significant results were obtained when comparing all the samples obtained from the study population, the results also indicated that in specific cases there were significant differences (> 50-150%) between the A and B samples. Comparison between collection procedures was inconclusive and further studies need to be conducted in order to confirm significant differences between the procedures.

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Chapter 7

APPLICATION OF DIFFUSION STUDIES ON THE *IN VITRO* RELEASE OF COCAINE AND ANHYDROECGONINE METHYL ESTER DEPOSITED IN ORAL MUCOSA INTO SYNTHETIC ORAL FLUID

7.1 INTRODUCTION

Drugs that are consumed orally are delivered into oral tissues and the systemic circulation and then subsequently release into oral fluid (OF) via passive diffusion through the oral mucosa (Ceschel et al. 2002, Huestis and Cone 2004). This transport (i.e. kinetics) of drugs across tissues are widely studied using *in vitro* permeability or diffusion studies (Nicolazzo and Finnin 2008). Permeability studies also allow the evaluation of the nature of the biological barrier (tissue). Studies developed in *in vitro* models offer advantages over *in vivo* models as variables such as temperature, pH and drug concentration can be easily controlled. Additionally, in cases where human tissue is not available, animal models can be used instead, which reduces cost and ethical considerations (Patel et al. 2012).

In vitro methods commonly involve the use of diffusion cells fitted with suitable membranes, e.g. porcine skin, that operate under atmospheric conditions. The most used diffusion cells are the static diffusion cells also known as Franz cells (Figure 7.1) (Nicolazzo and Finnin 2008, Brodin et al. 2010, Bartosova and Bajgar 2012, Sjögren et al. 2014, Castro et al. 2016).

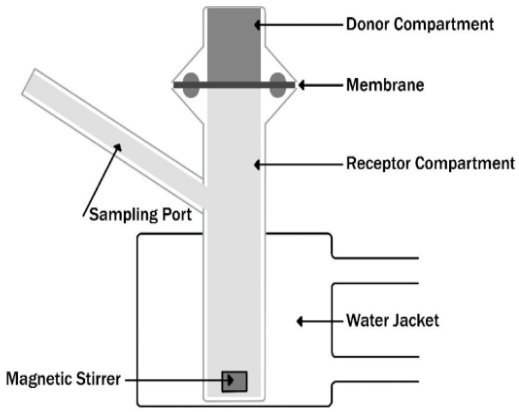

Figure 7.1 Franz type diffusion cell. (Bartosova and Bajgar 2012)

Diffusion studies using Franz cells (Figure 7.1) are normally conducted by applying a dose on the donor compartment and subsampling the diffused drug from the receptor compartment (as described in Section 1.9.2). However, in the *in vitro* model described in this chapter, the diffusion of cocaine and anhydroecgonine methyl ester (AEME) was monitored from the membrane into the donor and receptor compartments. Similar to the sampling of the process conducted using cell-based *in vitro* models (e.g. caco-2). In cell models, the membrane is initially impregnated with the drug and then the diffused drug is subsampled from the apical as well as the basolateral side of the well plate (Teksin et al. 2010, Yang et al. 2011, PermeGear Inc. 2014).

The use of porcine oral mucosa with non-keratinised epithelium, e.g. cheek offer higher permeability compared with keratinised epithelium, e.g. tongue tissue (Castro et al. 2016). Furthermore, the buccal mucosa which lines inside the cheek is more accessible and can be easily removed (Nicolazzo and Finnin 2008). Porcine oral mucosa has a similar physiological characteristics to the human oral mucosa such as the structure, morphology, composition and permeability (Squier 1991, Squier and Kremer 2001, Nicolazzo and Finnin 2008).

The main mechanism involved in the transfer of molecules across the oral mucosa can be described by mathematical models, e.g. zero and first order models (Chapter 1, Section 1.9.3) and was initially described by Fick's first law in 1855 (Fick 1855, 1995). Fick's law proposes the concept that a solute will move from a region of high concentration to a region of low concentration across a concentration gradient. Mathematical modelling can be fitted on experimental data to determine physical parameters, such as the drug diffusion coefficient (Dash et al. 2010) and describe the release of drugs at a slow zero or

first order rate or the release of an initial high amount of drug followed by a slow release of zero or first order.

This chapter describes the evaluation of the kinetics of release of cocaine and AEME into SOF, using an *in vitro* model that involved the use of Franz diffusion cells, porcine oral mucosa and SOF. The model presented in this chapter was developed to mimic the *in vivo* process, where drugs such as cocaine are initially absorbed into the oral mucosa following oral exposure (dose) and subsequently release into OF. By using Franz diffusion cells it was possible to measure the amount of cocaine/AEME being release from drug depots formed in the oral mucosa at controlled conditions of dosage, side of drug exposure, side of drug collection, temperature and area of diffusion (Ceschel et al. 2002, Nicolazzo and Finnin 2008, Bartosova and Bajgar 2012, Castro et al. 2016). Cocaine and AEME were used in this study because they form drug depots in the oral tissue and are subsequently released into OF (Chapter 3 and 6). Cocaine is the main analyte detected in OF following the oral consumption of crack-cocaine, cocaine base or coca tea and AEME is used as a biomarker for the consumption of crack cocaine (Kintz et al. 1997, Lewis et al. 2004). AEME is also present in coca tea (Chapter 6).

7.1.1 AIM AND OBJECTIVES

7.1.1.1 *Aim:*

This chapter aimed to measure the release kinetics of cocaine and AEME from drug depots into SOF using an experimental *in vitro* model.

7.1.1.2 *Objectives:*

- Assess the release of cocaine and AEME from drug depots formed in porcine oral mucosa into SOF using an experimental *in vitro* model.
- Evaluate the diffusion of cocaine and AEME from drug depots into OF using an experimental *in vitro* model.
- Determine the permeability of cocaine and AEME across porcine mucosal epithelium.

7.2 METHODS

7.2.1 Materials

Porcine cheeks were purchased from L F B Meats, Bournemouth, UK.

Synthetic oral fluid (SOF) was prepared using the Cozart biosciences protocol (2008) "Production of Synthetic Saliva" (Appendix A).

Analytical standards and reagents used for the analysis of SOF and tissue were purchased as described in Section 4.2.1.

TELOS® H-CX 130mg/3mL mixed-mode SPE columns were purchased from Kinesis (Cambridgeshire, UK).

Parafilm® M roll was purchased from Sigma-Aldrich (Pool, UK).

7.2.2 Instrumentation

Vertical Franz diffusion cell 11.28 mm x 6.5 mL Type B with an area available for diffusion of 1.01 cm^2 and receiver volume of 6 mL were purchased from Copley Scientific Ltd, UK.

Analysis of SOF samples was conducted using a LC-MS/MS system consisted of a tandem quadrupole mass spectrometer coupled to a Waters Acquity UPLC® system. Details on the LC-MS systems were described in Chapter 4 - Section 4.3.

The micrometer (Mitutoyo Series 293) used to measure tissue thickness was purchased from Mitutoyo UK Ltd (Liampshire, UK)

7.2.3 Preparation of homogenised tissue

Tissue homogenates were prepared as described in Section 4.4.1.

7.2.4 Solution preparation for transport studies

Diffusion studies were conducted using solutions of AEME at 5 µg/mL in 1.0M phosphate buffer saline solution (PBS) pH 7, AEME at 50 µg/mL in SOF, cocaine at 20 and 200 µg/mL in SOF and cocaine at 20 µg/mL in 1.0M PBS pH 7. These solutions were prepared by spiking SOF or PBS with the required amount of drug from 1 mg/mL stock solutions of AEME and cocaine. Concentrations of 5 µg/mL AEME and 20 µg/mL cocaine were selected based on the expected concentration of these analytes in a cup of coca tea. Concentrations of 6.2 ± 0.2 µg/mL AEME and 33.8 ± 0.8 µg/mL cocaine were obtained for a cup of coca tea (200 mL) using two coca tea bags (Table 6.1). Additionally,

high concentrations of AEME and cocaine were selected (50 and 200 µg/mL in SOF respectively) as ten times the concentration of AEME and cocaine in a cup of coca tea. These high concentrations were used to ensure sink conditions at the moment of developing the diffusion studies.

7.2.5 Membrane preparation

Fresh excised samples of porcine buccal mucosa were removed from pig's heads (Figure 7.2) using surgical scalpel and stored on PBS pH 7 and ice during transportation to the laboratory. Porcine buccal mucosa (cheek tissue) was used in this study because the buccal mucosa of pigs has similar structure, morphology and composition to the human buccal mucosa (Nicolazzo and Finnin 2008). All porcine mucosa tissue was cleaned with PBS pH 7 buffer and water to remove impurities such as blood, the residual water was immediately dried with a tissue. The mucosa was then cut with scissors to a suitable size to fit the Franz cell (pieces of approximately 2 x 2 cm). PBS was used as it has been reported that porcine buccal mucosa retained its integrity when stored with a preservative (PBS pH 7, Kreb's bicarbonate ringer solution, HEPES buffer or HBSS – Hank's balanced salt solution) at 4 °C for 24 hours (Kulkarni et al. 2010). Furthermore, it can also be stored for a more extended time at -20 °C following sectioning of the tissue without losing the tissue integrity (Michaud and Foran 2011).

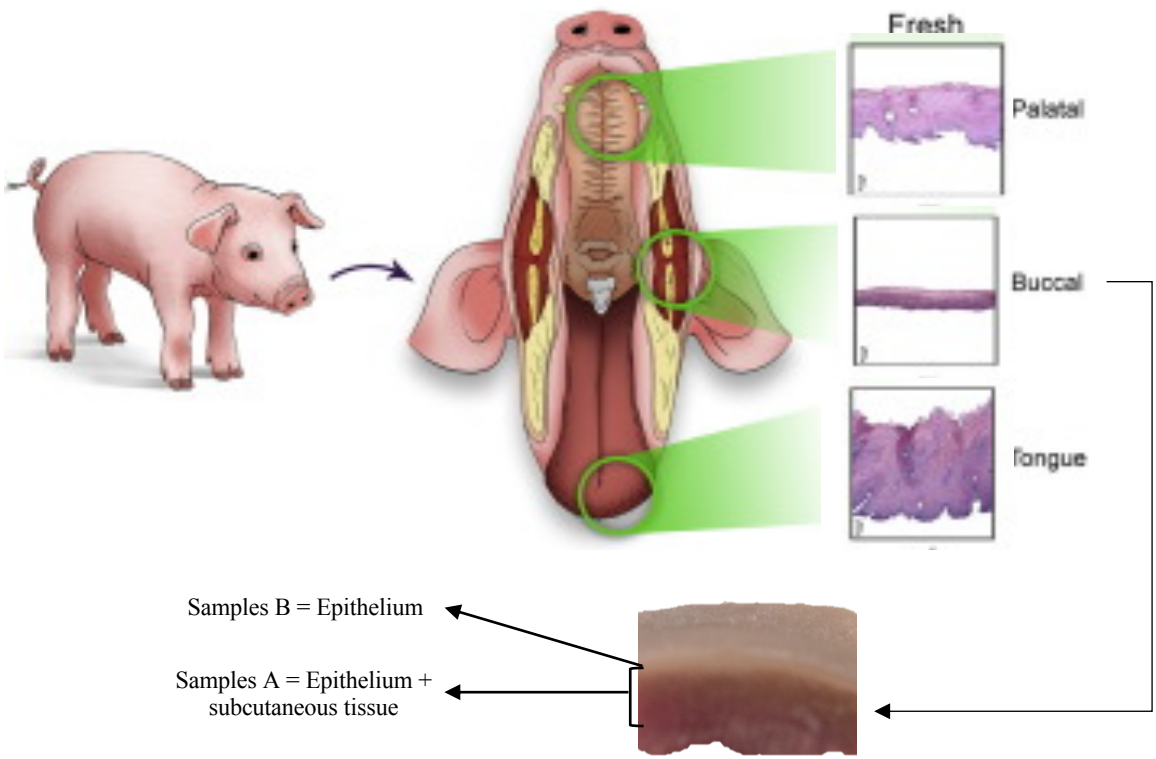

Figure 7.2 Diagram describing the process to obtain buccal mucosa samples. Porcine buccal tissue was excised from the cheek area of pig heads, then cut is sections of approximately 2 x 2 cm. Samples containing epithelium and subcutaneous tissue (A) and epithelium (B) were used for the diffusion strudies. Pigs head image from (Franz-Montan et al. 2016)

Membranes containing epithelium and subcutaneous tissue (A) and membranes containing epithelium (B) were used (Figure 7.2). The membranes A were carefully cut using a scalpel to obtain sections of 0.45-0.77 cm thickness. This thickness allowed the use of the epithelium, underlying connective tissue and some muscle tissue. The muscle tissue was included in the diffusion studies because drug depots are likely to be deposited within the muscle fibres as presented Section 3.3.2 and Chapters 8 and 9 from Reichardt (2014). The membranes B were obtained by removing the subcutaneous tissue (and most of the underlying connective tissue) using a scalpel to isolate the epithelium (Hoogstraate and Boddé 1993). Thickness of epithelium ranged from 0.12-0.17 cm. Mucosa thickness was measured before the experiment using a microtomer. The mucosa sections were immediately mounted on the diffusion cells or stored at -20 ºC until further use.

7.2.6 Release studies using the whole mucosa

7.2.6.1 *Conditioning of the diffusion cell*

Before the experiment, the fresh tissue or tissue section was thawed at room temperature and then mounted on the cells (Figure 7.2) with a 5 mm magnetic stirrer (600 rpm) in the receiver compartment. The top of the cell was positioned over the membrane, where the epithelium side faced the donor compartment and subcutaneous tissue, or submucosa side faced the receptor compartment (Figure 7.3).

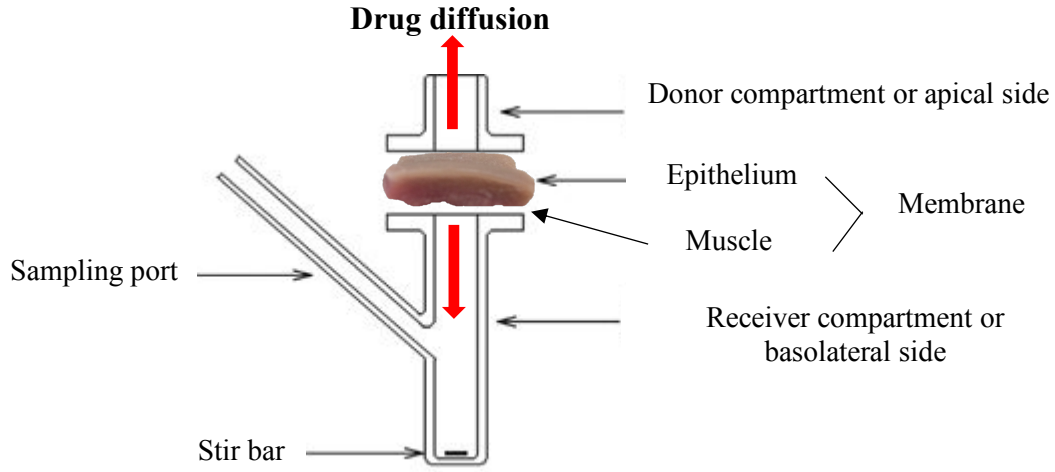

Figure 7.3 Diagram of diffusion using a Franz cell. Red arrows indicate the direction of the diffused drug. Drug can diffuse from drug depots formed in the mucosa tissue into SOF (Donor compartment) and PBS (receiver compartment).

The cells were sealed by wrapping parafilm around the two sections (donor and receiver compartments). Then, the receiver compartment was filled with pre-warmed phosphate buffered saline (1.0M PBS pH 7) (37°C). PBS is a physiological solution commonly used in permeability studies and is used to mimic the systemic circulation (Squier 1991, Salerno et al. 2010, PermeGear Inc. 2014, Castro et al. 2016). PBS at pH 7 simulates *in vivo* plasma pH (Castro et al. 2016). To ensure there was no leakage, the cells were inverted and visually inspected. One millilitre of SOF was then added to the donor

compartment. Each cell was submerged in a water bath at 37°C and cells were left to equilibrate for 30 minutes (Veuillez et al. 2002). During the experiment, the receptor compartment was stirred at a speed of 600 rpm. Stirring was conducted in order to ensure that there was no concentration gradient across the barrier (Brodin et al. 2010).

7.2.6.2 *Release studies*

After equilibration, the SOF from the donor compartment was removed and 1 mL SOF containing cocaine (20 or 200 µg/mL) at pH 7.3 or 6.3 and AEME (50 µg/mL) at pH 7.3 was applied for 10 min (Table 7.1). This procedure was conducted to allow the absorption of cocaine and AEME into the oral mucosa and therefore the formation of drug depots. The time of exposure was equal to the maximum time that participants took to drink a cup of coca tea (Chapter 6). The amount of drug absorbed by the mucosa was calculated by measuring the concentration of analytes in SOF before and after the dosing by LC-MS.

Table 7.1 Conditions evaluated in the release of cocaine and AEME from drug depots formed in oral mucosa into SOF.

Mucosa Membrane	Experiment number	Analyte concentration (ug/mL)		SOF pH	PBS pH
A	1	AEME	50	6.3	7
	2	COC	20	7.3	7
	3	COC	200	7.3	7
	4	COC	200	6.3	7
B	5	AEME	5	7.3	7
	6	COC	20	7.3	7

A: Whole porcine oral mucosa that included the epithelium and subcutaneous muscle, B: Porcine oral mucosa epithelium. AEME: Anhydroecgonine methyl ester, COC: Cocaine, SOF: Synthetic oral fluid, PBS: Phosphate buffer saline.

Following the dosage, seven washes of 1 mL control SOF were applied sequentially on the donor compartment for two minutes each to remove any excess of drug on the

epithelium. All washes were then stored at -20ºC for further analysis. Once the washes were collected, the PBS from the receiver compartment was removed and replaced with fresh pre-warmed PBS. The PBS solutions were later analysed for cocaine, BZE and AEME in order to identify if there was any drug being permeated into the receiver compartment from the donor compartment and/or drug depots formed in the mucosa. Similarly, the washes were analysed to confirm that the donor compartment was free of contamination.

In order to evaluate the release of cocaine and AEME from the drugs depots formed in the mucosa into the SOF, serial sampling of SOF was conducted from the donor compartment over a period of four hours. Samples of SOF (1 mL) were collected (at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210 and 240 min) and the equal volume of fresh pre-warmed control SOF was replaced after each withdrawal. The SOF was fully replaced at any time point to allow sink conditions (Brodin et al. 2010). Diffusion under sink conditions occurred when the amount of drug present in the receiver solution did not exceed 10% of the donor concentration (concentration of analyte in the mucosa). This allowed a concentration gradient where the diffusion per unit time (Jmax) was proportional to the thermodynamic activity (α_s) of the compound and not its concentration (Higuchi 1961). During the experiments, the receiver fluid was stirred with a magnetic rotor at a speed of 600 rpm and the cells were placed in a water bath at 37°C.

After the experiments finished, the mucosa tissues were carefully removed from the cells, homogenised and analysed for AEME, BZE and cocaine by LC-MS as described next.

Power analysis conducted on data (permeability coefficient values) obtained from the permeability of lidocaine hydrochloride across porcine oral mucosa (Franz-Montan et al. 2016) indicated that the sample size for *in vitro* studies using Franz cells and porcine oral mucosa is 1.4. This sample size was calculated using the Equation 6.1 and the following values: mean value of 0.96 ± 0.36 (n = 6), imprecision of 10%, significance level of 0.05 and power of 80% (Crawley 2005). Although a minimum value of one was obtained as sample size, it is common that the minimum value of replicates used in *in vitro* permeability studies be set at three or six (Salerno et al. 2010). In this thesis a minimum number of six replicates per experiment was used.

7.2.7 Permeability studies using porcine mucosa epithelium

This study was conducted to evaluate the effect of the epithelium on the mass transfer of cocaine and AEME from the PBS (basolateral side) to the SOF.

7.2.7.1 *Conditioning of the diffusion cell*

All cells were conditioned as mentioned in the section 7.2.7. After equilibration, the SOF and PBS were removed from the donor and receiver compartments.

7.2.7.2 *Permeability studies*

To evaluate the permeability of AEME and cocaine through the mucosa epithelium, the receiver compartment was filled with PBS pH 7.3 containing AEME and cocaine at 5 μg/mL and 20 μg/mL respectively. The donor compartment was filled with one millilitre pre-warmed SOF. Serial sampling of SOF was collected from the donor

compartment over a period of four hours. Samples of SOF were collected at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210 and 240 min. One millilitre of SOF was collected and replaced with one millilitre of fresh pre-warmed control SOF at the specific times. The SOF was fully replaced at any time point to allow sink conditions (Brodin et al. 2010). The minimum number of replicates per experiment was $n = 3$ (Salerno et al. 2010).

7.2.8 Quantitative analysis of synthetic oral fluid and porcine oral mucosa samples

All SOF and tissue samples were extracted and analysed for AEME, BZE and cocaine using the validated LC-MS method described in Chapter 4. All samples were analysed using calibrators and QCs prepared on the day of the analysis.

7.2.9 Release kinetics

Franz diffusion cells are practical and robust, but they do not mimic the salivary flow as efficiently (Castro et al. 2016). To overcome this drawback the volumes of the donor and receiver compartments should continuously be flushed with fresh solutions (mimicking a flow-through system), or by treating the obtained data using a set of equations that take the changing concentration gradient into account (Brodin et al. 2010).

To calculate the kinetics of release of cocaine into SOF, the cumulative amount of drug (Q) in the donor compartment released from the mucosa per diffusional area (A) was plotted against time (t). The profiles of cumulative amount of AEME and cocaine release from the drug depots formed in the mucosa at different intervals of time were fitted with various kinetic models. Table 7.2 summarise the kinetic models used to characterise the mechanism of drug release (Singh et al. 2017): Zero order, First order, Higuchi and

Korsmeyer Papas. The mass transfer coefficient was calculated from the slope of each curve. The flux of release (*J*) was calculated using the best fit with linearity of $R^2 > 0.95$ over at least five values at steady state.

Table 7.2 Mathematical models used for the calculation of the mass transfer coefficient.

Model	Equation	Units of mass transfer coefficient (K)
Zero Order	$Q_t = K_0 t + Q_0$	μg cm^{-2} h^{-1}
First Order	$lnQ = lnQ_0 - K_1 t$	h^{-1}
Higuchi	$Q = Q_0 + K_H\sqrt{t}$	$h^{-0.5}$
Korsmeyer - Peppas	$Q = Q_0 K_{KP} t^n$	Related to *n* value

Q: Amount of drug released; Q_0: initial amount of drug; t: Time; K_0: Kinetic coefficient zero -order; K_1: Kinetic coefficient first-order; K_H: Higuchi Kinetic coefficient; K_{KP}: Korsmeyer-Peppas kinetic coefficient; μg: micrograms; cm^{-2}: square centimetre; h: hour.

7.2.10 Statistical analysis

All data was presented as mean ± standard deviation. Kolmogorov-Smirnov test and Levene's test were used to check the normality and homogeneity of variance. Significant differences between experimental groups were evaluated using non-parametric Kruskal-Wallis H test (multiple comparisons) and Mann-Whitney U-test (two group comparison) (IBM SPSS Statistics version 23). Results with 2-tailed $p_s < 0.05$ were considered significant.

7.3 RESULTS AND DISCUSSION

7.3.1 Release studies

In order to assess the *in vitro* release of cocaine and AEME from drug depots into SOF, it was necessary to allow the formation of drug depots in the oral mucosa. Oral absorption

of cocaine hydrochloride and crack cocaine was reported to be rapid, as mean plasma concentrations were obtained immediately after crack smoking (Cone et al. 1994). Jenkins et al. (2002) also confirmed the rapid absorption of cocaine by reporting mean peak plasma concentrations after two minutes of smoking 40 mg of cocaine base. In this study, after ten minutes of exposure 4.8 ± 1.0 % (n = 6) of the cocaine dose (20 or 200 μg/mL) and 3.9 ± 0.4 % (n = 3) of the AEME dose (50 μg/mL) was absorbed by the mucosa. Quantification of the receiver compartment confirmed that no cocaine or AEME was permeated during the exposure period as concentrations of cocaine in PBS were < 2.3 ng/m and AEME was not detected at all times.

The release profile of cocaine and AEME from formed drug depots in the mucosa is illustrated in Figure 7.4. Both release profiles for cocaine and AEME in SOF showed an initial rapid release followed by slower release, similar to what was observed for the release of cocaine and AEME in OF following the ingestion/swirling of coca tea (Chapter 6). The initial increased rate seen could be explained if molecules were absorbed at different depth of the mucosa. In this scenario, the molecules closer to the epithelium would be more rapidly released into the SOF and molecules deeper absorbed move slower through the tissue towards the epithelium. This idea could be confirmed by the results presented in Chapter 3, where cocaine was detected at higher concentrations in sections closer to the epithelium. This gradient in concentration in porcine oral tongue tissue was also observed by Reichardt (2014, p 205-309).

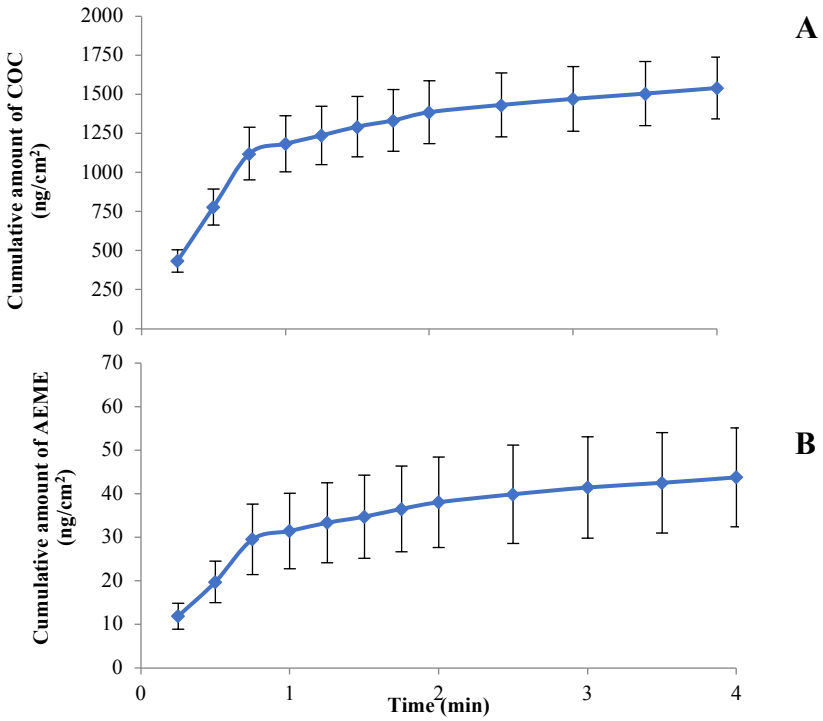

Figure 7.4 Cocaine (A) and AEME (B) release profile from drug depots in porcine oral mucosa (n = 3). Release experiments conducted using 20 and 50 µg/mL cocaine and AEME in SOF. Error bars represent the standard deviation of the data.

Additionally, the initial rapid mass transfer could also be attributed to the desorption of the cocaine and AEME molecules from the transcellular spaces of the epithelium, which are subsequently dissolved into the SOF. This process was considered to be less likely to occur as the surface of the mucosa was washed several times to eliminate any excess of compound on the surface of the epithelium. The final concentration of the washes were more than five times the concentration at time 15 min in each experiment. Mean concentration of cocaine and AEME in the last wash was 33.2 ± 3.8 and 3.4 ± 1.7 ng/mL respectively.

The profiles observed in all release studies were similar to the ones presented in Figure 7.4, with an initial rapid release followed by a slower release. The percentage cumulative amount of cocaine and AEME (mean values) obtained for all experiments is shown in Table 7.3. The percentage cumulative drug release was calculated using the total amount

of drug released into the SOF and the amount of drug that remained in the tissue after the experiment. These results showed that the amount of cocaine accumulated in SOF was higher for the release studies with higher dose (200 μg/mL) than with a normal dose (20 μg/mL). More than 20% of the total accumulated cocaine was released during the first 30 minutes. In comparison, release studies using a normal dose (20 μg/mL) showed that more than 20% of the cumulative cocaine was released after 150 minutes. The results obtained using AEME at a high dose (50 μg/mL) were similar to the results obtained with the high dose of cocaine as more than 20% of the cumulative AEME was released after 45 min.

Table 7.3 Percentage cumulative release of cocaine and AEME in SOF. Data expressed as mean values ± standard deviation.

Time (h)	% COC Cumulative drug release			% AEME Cumulative drug release
	A (n = 6)	B (n = 6)	C (n = 6)	D (n = 3)
0.25	6.1 ± 1.0	16.3 ± 2.5	11.3 ± 0.6	9.2 ± 1.8
0.50	9.7 ± 1.8	26.9 ± 3.5	20.3 ± 0.8	15.3± 2.7
0.75	12.1 ± 2.2	34.2 ± 2.5	29.2 ± 1.2	22.5 ± 3.8
1.00	14.1 ± 2.6	35.3 ± 2.3	30.8 ± 1.2	23.9 ± 4.0
1.25	15.8 ± 3.0	36.4 ± 2.2	32.2 ± 1.3	25.3 ± 4.1
1.50	17.1 ± 3.4	37.3 ± 2.1	33.7 ± 1.3	26.4 ± 4.2
1.75	18.3 ± 3.6	38.1 ± 2.0	34.7 ± 1.2	27.8 ± 4.3
2.00	19.4 ± 3.9	39.0 ± 1.9	36.1 ± 1.2	28.9 ± 4.6
2.50	20.9 ± 4.3	39.9 ± 1.8	37.4 ± 1.1	30.2 ± 5.0
3.00	22.1 ± 4.6	40.7 ± 1.6	38.4 ± 1.0	31.4 ± 5.1
3.50	23.2 ± 4.9	41.3 ± 1.5	39.3 ± 0.9	32.3 ± 4.8
4.00	24.1 ± 5.1	41.9 ± 1.4	40.3 ± 0.5	33.4 ± 4.4

A: SOF pH 7 and initial dose of 20μg COC; B: SOF pH 7 and initial dose of 200μg COC; C: SOF pH 6 and initial dose of 200μg COC, D: SOF pH 6 and initial dose of 50μg AEME; AEME: Anhydroecgonine methyl ester, COC: Cocaine, h: hour; n = number of experiments.

Statistical Kruskal-Wallis test indicated that there were significant differences in profiles between the samples within the same experiment ($p < 0.001$) and between the doses 20 and 200 μg/mL ($p < 0.001$). The significant differences inter-experiment could have been the result of the different amount of drug absorbed within the tissue.

In a separate experiment conducted on porcine tongue tissue (n = 3) it was observed that the concentration of cocaine absorbed or deposited in an individual tissue (subsamples n = 20) varied with a %RSD > 40%. Comparison between experiments indicated that there were significant differences between the concentration of cocaine in the tissue (Kruskal-Wallis test p <0.001) (Appendix G). These differences were attributed to the differences inherent to the specific section of the tissue as are the difference in thickness of the epithelium, membrane basal, amount of connective tissue and muscle fibres, which can vary significantly within the same specimen (Gómez de Ferraris and Campos 2002). Even though significant differences (p < 0.001) were obtained between the different profiles within the same release experiment and between doses, this appeared to not affect the diffusion coefficient of cocaine or AEME as will be discussed next.

7.3.1.1 *Kinetics of release*

Table 7.4 shows the values calculated for the flux and release coefficient for the different cocaine and AEME experiments (Experiments 1-4 from Table 7.1). The results indicated that the release flux of cocaine increased with the increase of concentration, which can be explained by the increased gradient in concentration. Surprisingly, a decrease in flux was observed when the pH of the SOF was decreased. At low pH, cocaine become more ionisable and therefore more hydrophilic, hence an increase in flux was expected at lower pH (Schramm et al. 1992). Nonetheless, the flux is directly related to the initial concentration of cocaine in the tissue and the thickness of the mucosa (Nicolazzo and Finnin 2008) and the diffusion coefficient obtained at the different pH values were similar as will be discussed next.

Table 7.4 Results for the release kinetics using various release models for cocaine and AEME.

Release kinetics		COC			AEME
		Dose 20 ng/mL	Dose 200 ng/mL		Dose 50 ng/mL
		SOF pH 7.3	SOF pH 7.3	SOF pH 6.3	SOF pH 6.3
Flux	J ($ngcm^{-2}h^{-1}$)	66.9	107.0	66.8	135
	R^2	0.96	0.94	0.96	0.98
Zero	K_0 (h^{-1})	0.0489	0.0992	0.0678	0.0836
	R^2	0.64	0.68	0.66	0.76
First	K_1 (h^{-1})	0.280	0.221	0.220	0.24
	R^2	0.75	0.54	0.53	0.62
Higuchi	K_H ($h^{-1/2}$)	244	631	378	19.1
	R^2	0.97	0.82	0.80	0.88
Kosmeyer-Peppas	n	0.463	0.402	0.403	0.430
	K_{KP}	292	1017	613	27.4
	R^2	**0.98**	**0.85**	**0.84**	**0.90**

AEME: Anhydroecgonine methyl ester, COC: Cocaine, h: hour; K_0: Kinetic coefficient zero -order; K_1: Kinetic coefficient first-order; K_H: Higuchi Kinetic coefficient; K_{KP}: Korsmeyer-Peppas kinetic coefficient; *n* = release exponent indicative of mechanism of release.

Following the analysis of release profiles with the mathematical models, it was noted that the best linearity was obtained using the Korsmeyer-Peppas model (Costa and Sousa Lobo 2001) and therefore the results obtained with this model were used for comparison of the release coefficient. The diffusion exponent *n* using the Korsmeyer-Pappas model confirmed the Fickian diffusion, e.g. drug release was dependent on concentration since *n* was less than 0.5 (Singh et al. 2017).

Statistical Kruskal-Wallis H test indicated that there were no significant differences in the release coefficient (K_H) within the same experiment (Experiment 1: H(5) = 5.0, p = 0.4 and Experiment 2: H(5) = 5.0, p = 0.4). Additionally, a Mann-Whitney U test indicated that there were significant differences between the release coefficient (K_H) for the two concentrations evaluated (20 and 200 μg/mL), U = 1.00, p = 0.005. These results were in agreement with the results obtained for the flux (J). The increase in release coefficient indicated that the release of cocaine from drug depots formed in the mucosa

into SOF was concentration dependent. This relation between the mass transfer and concentration was observed in a different study for the permeability of dexloxiglumide using a Caco-2 cell model (Tolle-Sander et al. 2003). Concentrations of 8 and 80 μg/mL dexloxiglumide in 10mM HEPES buffer provided permeability values (apical-basolateral direction) of 11.7 ± 0.6 cm/s and 17.3 ± 1.5 cm/s respectively. The concentration dependency in Tolle-Sander's study was attributed to the polarisability of the molecule and its preference to transport from the basolateral-apical side instead of the apical-basolateral direction.

In the present study, the preference in transport of cocaine across the mucosa from the apical to basolateral direction was evaluated partially, as sampling from the basolateral side of the cell was conducted only twice (before diffusion studies started and after they finished). This allowed the measurement of concentrations at the beginning and at the end of the experiments. The quantification of the receiver compartment at the end of the study indicated that no drug was released from the mucosa into the PBS buffer during the release studies at concentrations above the LOD (0.1 ng/mL) of the LC-MS method. Changes in K values were finally attributed to differences in concentration gradient between the mucosa and SOF as well as between regions of less concentration within the tissue (Brodin et al. 2010). It is possible that lower number of cocaine molecules deposited in the mucosa tissue (low concentration) could lead to an increase of regions with less concentration of cocaine, where the gradient in concentration takes place. The results presented in Chapter 3 suggested that cocaine was deposited in specific areas within porcine tongue tissue.

Since a minimum amount of cocaine was detected in the basolateral side, it can be implied that cocaine deposited in the mucosa was primarily diffused towards the apical

side of the mucosa and therefore towards the SOF. This dynamic suggested that the final concentrations of cocaine in OF might get affected as the amount of cocaine in OF could be the result of the cocaine transported from the blood circulation as well as the transport of cocaine deposited in the oral cavity. Therefore, it can be related to the high concentrations of cocaine presented in OF samples that various authors have reported (Kato et al. 1993, Kidwell et al. 1998, Bosker and Huestis 2009). The presented results also confirmed the slow release of cocaine into OF from drug depots (Chapter 6) and implied that the diffusion of cocaine decreased with the amount of cocaine present in the oral mucosa.

Statistical analysis using a Mann-Whitney U test (U = 1.0, $p = 0.2$) indicated that there were no significant differences between the release (K_H) of cocaine into SOF at pH 7.3 or 6.3. The K_{KP} values had n values below 0.5, (pH 7.3: 1017 h^{-1}; pH 6.3: 612 h^{-1}) which indicated that the transport of cocaine into SOF was concentration dependent. This result could suggest that variations in the pH of human neat OF (normal OF pH values range between 6.0 and 7.0) would not have any effect on the release of cocaine deposited in the mucosa tissue (Gjerde et al. 2010). It is important to note that changes in the pH of the OF could be related to changes in the production and flow rate of OF (Aps and Martens 2005), which could affect the diffusion of drugs.

The flux of AEME (135 ng/cm^2/h) was double the flux of cocaine (66.8 ng/cm^2/h; dose 200 μg/mL) at the same SOF pH 6.3 (Table 7.4). This result indicated that AEME was being released more rapidly than cocaine from the mucosa. However, a decrease of 20% of the total AEME and cocaine concentration was released after 45 and 30 min respectively. As per cocaine, the model that best described the release of AEME into SOF was the Korsmeyer-Pappas. This model provided an n value of less than 0.5. Therefore

the kinetics of AEME followed a Fickian diffusion (Singh et al. 2017). The values of K_{KP} obtained for AEME (27.4 $h^{-0.4}$) was 23 times lower than the value obtained for cocaine (612 $h^{-0.4}$). However, the initial amount of AEME absorbed in the mucosa (58 ng/g mucosa) was 26 times lower than the cocaine concentration (1512 ng/g mucosa). Therefore, it can be implied that AEME diffused into the mucosa with a similar rate as cocaine. Differences in diffusivity could be the result of the more lipophilic properties of AEME compared to cocaine since cocaine contain more hydrogen bond donor and acceptor (one and five respectively) than AEME (zero and three respectively) (Figure 1.2).

7.3.2 Permeability studies

The permeability across the porcine epithelium of the oral mucosa was measured in order to evaluate its influence in the transport of cocaine and AEME from drug depots formed in the muscle fibres within the oral mucosa into SOF. The permeability profiles for cocaine and AEME are shown in Figure 7.5. The profiles obtained revealed a constant release of drug over time.

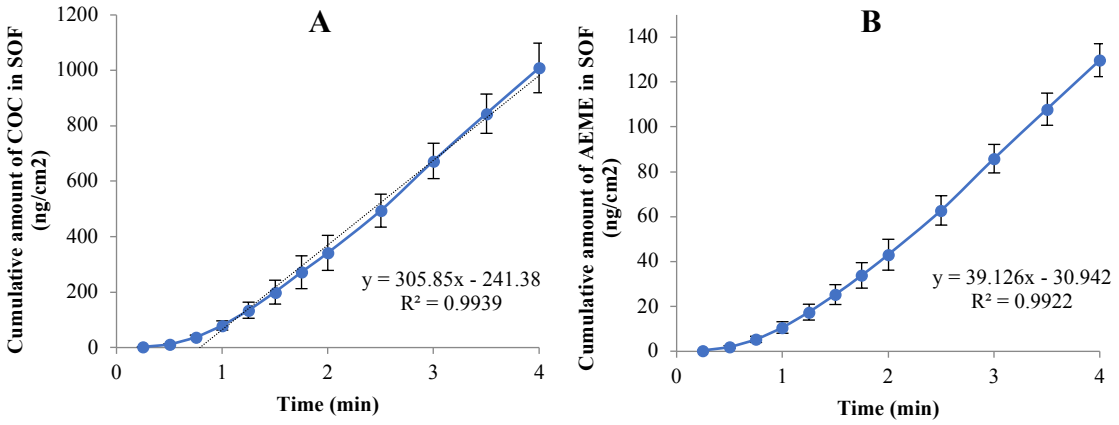

Figure 7.5 Cocaine (A) and AEME (B) permeability profiles across porcine mucosal epithelium. Permeability experiments conducted using 20 and 5 µg/mL cocaine and AEME in 1M PBS pH 7.

Drug permeation across the oral epithelium was described by a zero-order kinetic model (Cocaine $R^2 = 0.99$; AEME $R^2 = 0.99$). Since the amount of cocaine/AEME in the receiver solution (SOF) did not exceed 10% of the donor solution (PBS pH 7), it was assumed that the cumulative rate of drug passing through the epithelium was the steady-state flux. The results of the permeability studies are presented in Table 7.5. From this table, it can be noted that the flux was higher for cocaine than for AEME. This difference was the result of the drug concentration from the donor solution (Cocaine: 20 μg/mL in PBS pH 7 and AEME: 5 μg/mL in PBS).

Table 7.5 Permeability results of cocaine and AEME across porcine mucosal epithelium. (Mean value ± SD)

Drug	Flux (ng $cm^{-2}h^{-1}$)	Lag time (h)	K_0 ($cm^{-2}h^{-1}$)
COC (***n* = 3**)	305 ± 22	0.80 ± 0.8	0.0153 ± 0.001
AEME (***n* = 3**)	78.2 ± 2.8	0.77 ± 0.8	0.0156 ± 0.001

AEME: Anhydroecgonine methyl ester; COC: Cocaine; K_0: Diffusion coefficient; *n* = number of experiments.

The results showed that after 46 and 48 minutes a steady flux could be obtained for the release of AEME and cocaine into the SOF respectively. The lag time is the time that cocaine/AEME takes to permeate through the membrane and diffuse into the SOF with a stable flow rate (Bartosova and Bajgar 2012). A Mann-Whitney U test indicated that there were no significant differences between the lag time obtained for AEME and cocaine ($U = 4.0$, $p = 1.0$). Similarly, diffusion coefficients for AEME and cocaine did not have significant differences ($U = 4.0$, $p = 1.0$).

The diffusion coefficient using the flux at steady state was used to compare the diffusion coefficient obtained for the release studies and permeability studies. Mean diffusion coefficient for the release of cocaine (Experiment 2) was $K = 0.036 \pm 0.003$ cm^{-}

$^{2}h^{-1}$. The mean diffusion coefficient for the release of AEME (Experiment 1) was K = 0.051± 0.008 $cm^{-2}h^{-1}$. A Mann-Whitney U test indicated that there were no significant differences between the diffusion coefficient obtained for cocaine in the release studies compared with permeability studies (U = 1.0, p = 0.048). For the contrary, there were significant differences for the diffusion coefficient of AEME (U = 0.0, p = 0.1). The results obtained for the transport of cocaine across the whole mucosa and the epithelium indicated that the main barrier that cocaine has to pass is the epithelium and that other sections of the mucosa (such as lamina propria and submucosa; Figure 1.6) did not have a significant influence in its diffusion. The results for AEME, on the other hand, indicated that AEME diffused more rapidly through the epithelium than on the whole mucosa. This result could be attributed to the lipophilic nature of AEME and its potential bonding to lipids present in the mucosa such as the connective tissue.

Even though the stability of cocaine in both SOF and PBS solutions was confirmed in Chapter 5, the breakdown product of cocaine (BZE) was monitored in both release and permeability studies in order to confirm that no degradation of cocaine took place. The percentage of BZE was less than 5% the concentration of cocaine during the length of the study, with concentration of BZE in SOF increasing as the cocaine concentration increased. Mean percentages of BZE in experiments developed with cocaine (Experiments 2, 3, 4 and 6 as indicated in Table 7.1) were 2.8 ± 0.3, 3.4 ± 0.6, 2.21 ± 0.1 and 4.6 ± 0.7% respectively. The initial concentration of BZE in the solutions of cocaine were 1.7% for solutions at 20 and 200 μg/mL in SOF and 7.5% for the solution of 20 μg/mL cocaine in PBS (Permeability studies). Since the percentages of BZE did not vary more than 20% from the initial concentration of cocaine it could be confirmed that cocaine did not degraded during the release and permeability studies.

During the development of the release and permeability studies the viability of the oral mucosa was not monitored because of limitations on the instrumentation required for the analysis. However, Kulkarni et al (2010) reported that porcine oral mucosa retained its integrity when stored in PBS for up to 24 hours. The fact that the release and permeability studies were conducted within 24 hours after excision of the oral mucosa suggested that the mucosa retained its integrity during the length of the study.

The diffusion of cocaine by active transport was considered irrelevant as the principal mechanism of diffusion of drugs in the oral mucosa has been reported to be the passive diffusion (Squier 1991, Brodin et al. 2010, Bartlett and van der Voort Maarschalk 2012, Bartosova and Bajgar 2012, Frank and Hand 2014). Bhat et al. (2001) showed that cocaine transported across a T-84 epithelial cell line (an *in vitro* method to measure permeability across the intestinal wall) from luminal to serosal side of the intestine by simple diffusion. Bhat's study also indicated that receptor-mediated transport might not take place as there were no significant differences in the diffusion between the time periods of exposure (30 and 60 minutes) at different dosages of cocaine (100-800 ng) (Bhat et al. 2001).

7.4 CONCLUSIONS

The *in vitro* model evaluated in this chapter allowed the successful measuring of the release of cocaine and AEME from drug depots and the evaluation of the permeation of cocaine and AEME across oral mucosa. The results indicated that after ten minutes of exposure 4.8 ± 1.0 % of the cocaine dose (20 or 200 μg/mL) and 3.9 ± 0.4 % of the AEME dose (50 μg/mL) was absorbed by the mucosa. Furthermore, it was observed that cocaine and AEME did not permeate the oral mucosa during the exposure period.

The release profiles for both cocaine and AEME were similar in all experiments and showed an initial rapid release followed by a slower release. The initial increased rate was explained if molecules were absorbed at different depth of the mucosa and so molecules closer to the epithelium would be more rapidly released into the SOF at the same time molecules deeper absorbed into the tissue would move slower through the tissue towards the epithelium.

The Korsmeyer-Peppas model was used to evaluate the kinetics of release because this model provided the best correlation coefficient (R^2 ranged 0.85-0.99 for all experiments). The diffusion exponent *n* confirmed Fickian diffusion in all cases (Singh et al. 2017). Statistical analysis showed no significant differences in the release coefficient (K_H) within the same experiment K_H and between the K_H values obtained at pH 7.3 or 6.3. However, significant differences were observed between the K_H values of the two concentrations evaluated (20 and 200 μg/mL; $p = 0.005$). Additionally, it was observed that more than 20% of the total accumulated cocaine was released during the first 30 minutes (200 μg/mL as initial dose) in comparison to 150 minutes (20 μg/mL as initial dose). The increase in release coefficient indicated that the release of cocaine from drug depots formed in the mucosa into SOF was concentration dependent. Comparison between the release coefficient of AEME and cocaine showed that AEME ($K = 0.051 \pm 0.008$ $cm^{-2}h^{-1}$) diffused into the mucosa with a similar rate as cocaine ($K = 0.036 \pm 0.003$ $cm^{-2}h^{-1}$).

The results obtained in this study suggested that cocaine and AEME deposited in the mucosa diffused towards the apical side of the oral mucosa as no drug was detected in the receiver solution located on the basolateral side. This was an important finding because this factor could have an effect on the final concentrations of cocaine and AEME

in OF and therefore in OF cocaine testing. If the molecules of cocaine and AEME preferred to transport towards OF, the molecules coming from the blood into the OF would further affect this transport. The amount of cocaine released into the OF could therefore be the result of the cocaine/AEME transported from the blood circulation as well as the transport of the drug deposited in the oral cavity. What is more, cocaine and AEME from drug depots would not be transported into the systemic circulation increasing the concentration of these drugs in blood.

Permeability profiles for cocaine and AEME revealed a constant release of drug over time. Drug permeation across the oral epithelium was described by a zero-order kinetic model (COC R^2 = 0.99; AEME R^2 = 0.99). The results showed that after 46 and 48 minutes a steady flux could be obtained for the release of AEME and cocaine into the SOF respectively and no significant differences between the lag time were obtained for AEME and cocaine (p = 1.0). Similarly, diffusion coefficients for AEME and cocaine did not have significant differences (p = 1.0).

The results obtained for the transport of cocaine across the whole mucosa and the epithelium indicated that the main barrier to cocaine was the epithelium and that other sections of the mucosa (such as lamina propria and submucosa) did not have a significant influence in its diffusion. No significant differences were obtained between the diffusion coefficient in the release studies compared with permeability studies for cocaine (p = 0.048). The results for AEME, on the other hand, indicated that AEME diffused more rapidly through the epithelium than on the whole mucosa. This result could be attributed to the lipophilic nature of AEME and its potential bonding to lipids present in the mucosa such as the connective tissue. This was supported by the significant differences (p = 0.1) obtained between the diffusion coefficient in the release study and the permeability study.

This differences in release rate suggested that the windows of detection for AEME will be shorter than for cocaine in OF, which will therefore have an impact on the times of detection of these drugs in OF testing.

Chapter 8

CONCLUSIONS, RECOMMENDATIONS AND FUTURE WORK

8.1 CONCLUSIONS

The correct interpretation of results from the analysis of drugs in oral fluid (OF) is essential for the identification of drug abusers in different scenarios such as workplace drug-testing programs and driving under the influence of drugs (DUID). Interpretation from OF drug testing is generally difficult to achieve because the concentration of drugs (e.g. cocaine) can vary significantly in OF compared with blood samples (Wolff et al. 2017). Since drugs in OF have similar detection times to those found in blood, drug detection in OF could indicate recent use and therefore be correlated to the effect that the drug might have on the donor at the time of OF sample collection. As such, one of the major challenges in OF drug testing is to understand the high concentrations of drugs in OF which can be evaluated by studying the kinetics of release of drugs from oral drug depots into OF. Drugs consumed orally are likely to form drug depots in the oral cavity (Huestis and Cone 2004), and these depots might have the potential to increase the concentration of drugs in OF.

Cocaine was the drug of choice in investigating drug release from drug depots into OF because it is one of the most abused drugs worldwide (UNODC 2017), it is commonly detected at high concentrations in OF (Jenkins et al. 1995, Strano-Rossi et al. 2010, Reichardt 2014) and form oral depots in oral tissues (Reichardt 2014). The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported cocaine as the second most widely used drug of abuse in the UK and Europe (EMCDDA 2017) and the United Nations Organisation for Drug Control (UNODC) reported that approximately 17 million consumed cocaine in 2015 (UNODC 2017). Concentrations of cocaine in OF have been reported to be higher than in plasma (S/P > 3) (Jenkins et al. 1995, Scheidweiler et al. 2010, Fiorentin et al. 2017). Following crack-cocaine smoking cocaine can be found

at concentrations up to 3 μg/mL (Kato et al. 1993, Cone 2012), and after drinking of coca tea up to 9 μg/mL (Jenkins et al. 1995, Strano-Rossi et al. 2010, Reichardt 2014). Other authors have also reported the detection of cocaine in OF after nasal insufflation of cocaine base (Javaid et al. 1983, Cone et al. 1997, Jufer et al. 2006). Furthermore, cocaine and cocaine derivatives (cocaine metabolites and other products such as anhydroecgonine methyl ester AEME) form oral depots in oral tissues and these depots could subsequently be released into OF increasing their concentration in OF (Reichardt 2014).

The research presented in this thesis enhanced the current knowledge on the release of drugs from drug depots into OF and contributed to the interpretation of results from OF testing, by evaluating the elimination profile and times of detection of cocaine and cocaine derivatives (AEME, benzoylecgonine BZE and ecgonine methyl ester EME) in human OF samples. The research also addressed some of the processes surrounding the absorption and subsequent release of drugs from oral drug depots into OF, highlighting the differences between ingestion and swirling of coca tea (Chapter 6). Furthermore, the release kinetics of drugs (cocaine and AEME) from drug depots into OF were predicted using an *in vitro* model by measuring the diffusion and permeability of these drugs across porcine oral mucosa (Chapter 7).

The experiment design and the interpretation of results were aided by monitoring the changes in the concentration, i.e. stability of analytes in buffered OF (BOF), synthetic OF (SOF) and porcine oral mucosa under different storage temperature and time (Chapter 5). Quantitation of cocaine and cocaine derivatives in BOF, SOF and porcine oral mucosa was achieved by using two Liquid Chromatography – Mass Spectrometry (LC-MS) validated methods (Chapter 4). Analytical approaches were not only evaluated using classical techniques such as LC-MS, but also more modern techniques like Raman

spectroscopy. Raman spectroscopy and more specifically Surface Enhanced Raman Spectroscopy (SERS) was used as an alternative technique to evaluate the detection of cocaine in SOF and porcine oral tissue (Chapter 2 and 3). The SERS analysis was achieved by using substrates along with handheld Raman spectroscopy (Chapter 2), portable Raman spectroscopy and Raman microscopy (Chapter 3).

Contribution to knowledge was achieved by investigating the presence of cocaine and cocaine derivatives from oral drug depots into OF, more specifically analyte concentration, release profile and time of last detection. Previous research on the release of cocaine from oral depots reported that only cocaine and BZE were detected for up to one hour following the consumption of a cup of coca tea (Reichardt 2014). However, the present research showed that cocaine, BZE and other cocaine derivatives could be detected in OF for up to four hours following ingestion or swirling of coca tea. The previous derivatives included AEME which could be detected in OF (LOQ: 2 ng/mL) for up to one hour and EME which could be detected up to three hours (LOQ: 4 ng/mL). Cocaine and BZE were detected in OF giving positive results at the EWDTS (8 ng/mL for cocaine and BZE) for up to a period of three and four hours respectively. The detection of AEME in biological samples (OF and urine) has been mentioned in six studies of consumption of coca leaves (ElSohly et al. 1986, Engelke and Gentner 1991, Jenkins et al. 1996, Mazor et al. 2006, Strano-Rossi et al. 2008, Reichardt 2014), however, only its random detection was reported and no release profile had been investigated.

Comparison of the release profiles of cocaine and its derivatives (AEME, BZE and EME) in OF between participants that ingested and swirled coca tea showed differences in absorption of these drugs into the oral cavity and their release into OF. Participants that swirled coca tea showed peak analyte concentrations two to seven times higher in OF

compared with the participants that ingested the tea (Tables 6.5, 6.7, 6.9 and 6.11) during the first hour following dosage. The higher concentration of cocaine and cocaine derivatives in OF among participants that ingested and swirled coca tea suggested that a higher amount of drug depots were formed in individuals that swirled the tea because they retained coca tea in the mouth for a longer time (30-60 seconds). Participants who ingested the tea kept it in the mouth for few seconds before swallowing the tea. These drug depots were formed and released over a period of one to three hours.

The drug release was not consistent for all the evaluated analytes and it also varied between analytes depending on the release profile and metabolism (Figure 8.1). AEME and EME confirmed being released from oral drug depots because they showed positive testing (LOQ 2-4 ng/mL) in participants that swirled a cup of coca tea for a longer period of time than the participants that ingested the tea. On the contrary, cocaine and BZE showed that they were detected in OF for longer times at the EWDTS cut off (8 ng/mL) in the participants that ingested the tea.

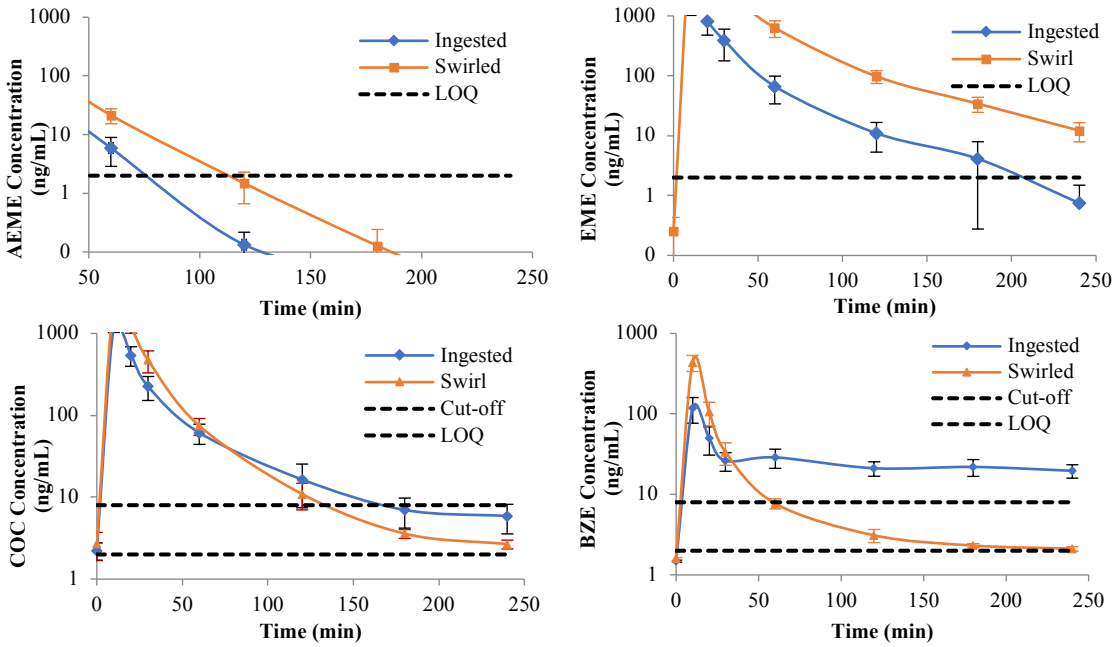

Figure 8.1 Times of last detection for cocaine, AEME, BZE and EME in OF following the ingestion or swirling of a cup of coca tea (Chapter 6). Bars are standard deviation. Dotted lines indicate the following cut-off concentrations: Limit of quantification (LOQ) of 2 ng/mL for cocaine, BZE and AEME, and 4 ng/mL for EME. EWDTS of 8 ng/mL for cocaine and BZE.

Taking into account the high BZE to cocaine ratios (BZE/COC) obtained by the drinkers, it can be suggested that cocaine and BZE were not only released from drug depots but from the systemic circulation when coca tea was ingested. The increase in BZE/COC ratio over time (Figure 6.4) was the outcome of the discrepancies observed between the last time of detection of drinkers and swirlers. Comparison between the time of last detection for all analytes suggested that differences in time were the result of the concentration of analytes in the oral depots or that analytes could have been released into OF at different rates.

During the development of the *in vivo* study the amount of analytes present in a single dose of coca tea was determined. Quantitative analysis of a methanolic extract of coca leaves and an aqueous infusion of coca leaves, i.e. coca tea demonstrated that as well as cocaine, AEME, BZE and EME were present in a cup of coca tea and the plant material (Table 6.1). These results suggested that all analytes present in the coca tea including cocaine, AEME, BZE and EME could be absorbed into the oral tissues forming drug depots in the oral cavity. Hence confirming that cocaine, AEME, BZE and EME could be detected in OF samples following the ingestion/swirling of coca tea as a result of oral absorption/contamination.

The presence of AEME in the coca plant material (extraction with methanol at room temperature) confirmed that AEME was inherent in the plant (*E. novogranatense* var. *nov*). For instance, the presence of AEME in the coca tea was the result of the extraction of AEME from the coca plant and not the result of thermal degradation of cocaine during the tea preparation. These findings are of importance for drug-testing because AEME, BZE and/or EME could be detected in biological samples including OF following the consumption of coca leaves, wrongly indicating the previous use of crack-cocaine or

cocaine base. Furthermore, it is possible that individuals that consume coca products could test positive for AEME, BZE and/or EME as well as cocaine. The fact that the composition of the coca tea varies depending on the origin of the coca plant (Casale et al. 2014), implies that the consumption of coca tea from different species of the coca plant could impact OF drug testing differently to what was described in this thesis.

In addition to the *in vivo* study of the release of cocaine and cocaine derivatives into OF, the analysis of paired OF samples collected simultaneously from different sides of the mouth (right and left) was studied. The results showed that overall there were no significant differences between the mean concentration of cocaine and cocaine derivatives from OF samples. However, differences from paired samples across the collected samples showed a distribution of more than one standard deviation for 15-24% of the samples. This indicated that in specific cases there were significant differences of more than 50% between the A and B samples.

In the absence of blood testing, the results of the *in vivo* study were confirmed using an *in vitro* model. The *in vitro* model (Chapter 7) involved using Franz diffusion cells, SOF and porcine cheek tissue under controlled conditions of dose, temperature, time of exposure and area of exposure (Ceschel et al. 2002, Nicolazzo and Finnin 2008, Bartosova and Bajgar 2012, Castro et al. 2016). The results of the *in vitro* study confirmed the outcome of the *in vivo* study in relation to the release of cocaine and AEME from oral drug depots into SOF. In agreement with the *in vivo* study, the *in vitro* study demonstrated that cocaine and AEME were deposited into the oral mucosa after exposure to the drug and subsequently released into OF over a period of four hours. In this respect, the amount of drug absorbed into the oral mucosa was proportional to the dose administered. Moreover, cocaine and AEME release into OF was dependent on the concentration of the

analyte in oral tissues (Singh et al. 2017), which confirmed Fick's law diffusion (Fick 1855).

The release profiles obtained for both cocaine and AEME using the *in vitro* study were similar to those obtained for the release of cocaine and AEME following exposure to coca tea. The release profiles showed that cocaine and AEME were released into OF at different rates, with molecules of analyte being more rapidly released during the first minutes after dosage. Higher concentrations of analytes were obtained in the *in vivo* study immediately after dosage as a result of oral contamination. The differences in release rate could have been the result of molecules of cocaine and AEME being absorbed at different depth in the oral mucosa. If molecules were absorbed at different depths, it is possible that molecules closer to the epithelium would be more rapidly released into the SOF than the molecules that were deeper absorbed, which moved slowly through the tissue towards the epithelium. This postulation was supported by the fact that cocaine was detected at higher concentrations in sections closer to the epithelium using Raman microscopy (results presented in Section 3.3.2) and immunohistochemistry staining (Reichardt 2014).

The higher flux and diffusion coefficient values obtained for cocaine and AEME across oral mucosa (Table 7.5) indicated that AEME diffused more rapidly through the epithelium than the whole mucosa. The diffusion coefficient for the transport of cocaine across whole mucosa (epithelium and muscle) were significantly different from the values obtained across mucosal epithelium, which indicated that the main barrier to cocaine transport was the oral epithelium. For the contrary, no significant differences were obtained between the diffusion coefficient of AEME across whole mucosa and epithelium, which indicated that AEME diffused similarly across both barriers. These results showed that the diffusion of drugs across oral mucosa was principally driven by

the physicochemical properties of the drugs, which confirmed the hypothesis that the mechanism of release of drugs from oral tissues into oral fluid was similar to the mechanism of action of drugs crossing the BBB (Section 1.10). Johnson et al. (2016) reported that the mechanism of diffusion of drugs (cocaine) across BBB depends on the structure and the hydration of the molecule.

The fact that cocaine and AEME were detected in the receiver compartment (and not in the donner compartment) indicated that the diffusion of cocaine and AEME deposited in the oral mucosa was higher towards the epithelium (apical side of the oral mucosa) rather than to the basolateral side (PBS) independently from the pH of the OF. This result indicated that higher concentrations of cocaine and AEME could be expected on the apical side of the cell (OF) than in the basolateral side (PBS). Since the transport of cocaine and AEME across the oral mucosa is higher towards the apical side of the mucosa where the OF is, there is a less probability that cocaine gets transported to the systemic circulation. This was an important finding as drugs such as cocaine and derivatives from drug depots could increase the concentration of drugs in OF and therefore affect OF drug testing. Furthermore, drugs (cocaine and derivatives) from drug depots might not be transported/released into the systemic circulation indicating that the concentration of drugs in blood would not be increased as a result of diffusion of drugs from drug depots. If a drug, e.g. cocaine is not transported from drug depots into the systemic circulation, this drug is not likely to contribute to the effect of the drug on an individual, e.g. psychoactive effect.

LC-MS was selected as the method to be implemented for the quantitative analysis of samples, as this is regarded a golden standard method for analysis of drugs in biological samples (Drummer 2006, Cooper et al. 2010, EWDTS 2015). LC-MS methods used in

this research were successful in quantifying cocaine and cocaine derivatives in multiple matrices (BOF, SOF and porcine oral tissue). These methods were validated according to the Scientific Working Group for Forensic Toxicology (SWGTOX 2013) and the Forensic Science Regulatory code of practice and conduct (Forensic Science Regulator 2017).

The LC-MS methods were also successful in monitoring the release of cocaine and derivatives from oral drug depots into OF/SOF and their stability in BOF, SOF and porcine oral tissue. The stability studies indicated that the length of storage and the storage temperature influenced the stability (concentration) of cocaine and cocaine derivatives in BOF, SOF and porcine oral tissue. The concentration of analytes decreased (cocaine, CE and EME) or increased (BZE) significantly after few days of storage in samples stored at room temperature or up to 37 °C. In particular, concentrations of cocaine and BZE in all matrices as the decrease in cocaine concentration increased BZE concentration, which is the result of cocaine degradation, i.e. hydrolysis of cocaine. Although the concentration of cocaine, CE and EME in BOF decreased as a result of degradation into BZE, norcocaethylene and ecgonine respectively, only the increase in concentration of BZE was evaluated in this study. The outcome of the stability study suggested that samples should be stored at low temperatures (-20 °C) in order to avoid degradation of cocaine and derivatives in any of the matrices (BOF, SOF or Tissue). This is because long transportation times and changes in ambient temperatures could lead to degradation of cocaine and cocaine derivatives in the samples. In conclusion, these results aided in the design of the *in*-vivo and *in-vitro* studies by identifying the best storage conditions of the samples and the implications that the length of storage (transportation of samples from Colombia to the UK) could have in the concentration of cocaine and derivatives in the samples.

Handheld and portable SERS analysis detected and quantified cocaine in SOF at concentrations above 100 μg/mL (range 0.1 – 1 mg/mL) using gold and/or silver substrates. The data demonstrated that silver nanoparticles (NPs) synthesised by the method of hydroxylamine reduction (Lee and Meisel 1982, Feng 2015) provided a more reliable SERS method than the commercial substrates. The study demonstrated that cocaine could be detected in SOF using SERS, however, the low sensitivity of the SERS method did not allow the detection of cocaine in SOF at physiological levels and therefore this method could not be applied in the monitoring of drug release in OF.

The SERS analysis of cocaine using silver NPs obtained by hydroxylamine reduction and handheld/portable Raman spectroscopy demonstrated that this technique has potential uses in on-site drug testing. However, the sensitivity of the method needs to be improved in order to be applied in the practice of human performance forensic toxicology and criminal forensic toxicology. For example, the cut-off values of cocaine in OF have been reported at concentrations of 8 ng/mL for workplace drug testing (EWDTS 2015). In order to increase the sensitivity of the method, more sensitive Raman spectrometers should be used along with SERS substrates of higher activity (high number of hot spots).

Raman microscopy allowed the detection of cocaine in porcine tissue at physiological concentrations (0-3 μg/mL) without the use of SERS substrates. The high sensitivity of Raman microscopy compared with conventional Raman spectrometers (Turrell and Corset 1996) allowed the detection of cocaine in oral tissue in different sections of the tissue. The optimisation of the method confirmed that lasers with low energy (532 nm) provided higher sensitivity for the detection of cocaine. Variation in the intensity of Raman scattering of cocaine at different positions of the tissue supported the idea of

formed drug depots and indicated that cocaine was absorbed/deposited in specific areas within the tissue.

In summary, the research findings presented in this thesis contributed to the understanding of the release of drugs from drug depots by investigating the kinetics of release of cocaine and cocaine derivatives from drug depots using an *in vivo* and an *in vitro* model. This research confirmed that cocaine and cocaine derivatives formed oral depots into the oral tissues and suggested that higher amounts of drug depots can be formed when the oral cavity is exposed to the drug for an extended period of time. The results presented in this thesis showed that cocaine and derivatives could be released from drug depots at different rates over an extended period of time. Diffusion studies of cocaine and AEME across oral mucosa confirmed that AEME diffused more rapidly into OF than cocaine and indicated that these drugs were more likely to diffuse into OF than the systemic circulation. The studies described in this thesis suggested that the mechanism of release of drugs from drug depots into OF depend on the physicochemical properties of the drug. The higher amount of oral drug depots (cocaine and cocaine derivatives) increases the time of detection windows of these drugs in OF which affect OF drug testing and its applications to workplace drug-testing programs, driving under the influence of drugs (DUID), drug-treatment settings and the prison service where drugs of abuse are tested. Further studies need to be conducted with other drugs of abuse to identify the rates of elimination of drugs from drug depots and the effect these have in their time of detection windows in OF.

8.2 RECOMMENDATIONS

The positive detection of cocaine, BZE, AEME and EME in OF and the time that these analytes (30-120 minutes) could be detected in OF suggested a recommendation for regulatory agencies about the time of sample collection in countries where coca tea drinking is legal (Colombia, Peru, Bolivia and Argentina). The recommendation would be to wait at least three hours post administration before OF samples are collected to avoid misinterpretation of results from OF cocaine testing as positive testing could be the result of oral contamination.

Careful considerations need to be taken on OF cocaine testing in countries in South America where coca tea is frequently consumed because of the higher probability of detection of cocaine, AEME, BZE and EME in OF samples as a result of coca tea drinking. The positive testing of AEME in OF following ingestion/swirling of coca tea wrongly indicated the previous use of "crack" cocaine and showed that coca tea consumption has a high impact in OF drug testing. Furthermore, the positive detection of cocaine and cocaine derivatives following ingestion and swirling of coca tea present an essential finding for local authorities in countries where OF drug testing has been implemented (Finland, Sweden, Norway, the UK and the USA) (Wille et al. 2009, Chu et al. 2012, Vindenes et al. 2012), and where products of the coca plant are illegal consumed. This because positive cocaine drug testing could be the result of coca tea exposure and not the consumption of cocaine base or crack cocaine. The increase in the importation and illegally purchased from the internet of commercial products of the coca plant, e.g. coca tea in European countries and the USA (De la Cal 2016, Transnational Institute 2017, SERVINDI 2018), increases the probability of consumption of coca tea products, which could affect OF cocaine testing in these places. The research suggested

the inclusion of other biomarkers such as cuscohygrine and/or hygrine in OF drug testing by regulatory agencies to confirm the presence of AEME from coca tea drinking and to discriminate between coca tea/coca products consumption and drug abuse (Rubio et al. 2013).

Careful considerations should be taken by regulatory authorities and prosecutors for the equivalence of the A and B samples when two samples have been collected simultaneously. Significant differences in analyte concentration were obtained from the analysis of paired OF samples collected simultaneously from different sides of the mouth (right and left) for approximately 20% of the samples collected (n = 119). This indicated that in specific cases there were significant differences (> 50-150%) between the A and B samples. Which could result in invalidation of results as false positives or negatives could be obtained. In cases where the volume of OF collected is not a limitation it could be recommendated that sample collection be conducted using a single collection and subsequently splitting the sample to obtain the A and B samples. However, if two samples need to be collected, it is important that the devices and extraction procedures be optimised in order to decrease the discrepancies in results.

The outcome of the stability study of cocaine and cocaine derivatives in BOF, SOF or Tissue suggested that samples should be stored at low temperatures (-20 °C) in order to avoid degradation of cocaine and derivatives in any of the matrices. The data from this study suggested that caution should be applied to the interpretation of results in samples stored at room temperature or up to 37 °C as concentrations of analytes decreased (cocaine, CE and EME) or increased (BZE) significantly after few days of storage. In particular, concentrations of cocaine and BZE in all matrices as the decrease in cocaine

concentration increased BZE concentration, which is the result of cocaine degradation, i.e. hydrolysis of cocaine.

The results presented in Chapter 3 proved that Raman microscopy could be used for the detection of cocaine in biological tissues at physiological concentrations without the use of SERS substrates (Raman enhancers). These results implied that Raman microscopy could also be used to detect cocaine in OF samples at concentration in the nanogram level. The implementation of alternative techniques, i.e. Raman microscopy in drug testing could be beneficial for the accredited laboraties as this could reduce the time and cost of analysis.

8.3 FUTURE WORK

The *in vivo* study evaluated the detection window of cocaine and cocaine derivatives (AEME, BZE and EME) in OF employing a single dose administration of coca tea. However, in the South American countries, coca leaves are consumed multiple times a day. It is therefore essential to further assess the windows of detection of cocaine and derivatives in OF after multiple dosages of coca tea as the windows of detection are likely to vary with the dosage (Coe et al. 2018).

More research should be conducted for the presence of cocaine and cocaine derivatives in biological samples including OF following consumption of coca products. There are many products from the coca plant (coca leaves for chewing, coca flour and coca rum) that are available in the market and are being consumed as much as the coca tea in the South American countries. The consumption of these products could result in positive testing for cocaine in OF and therefore interfere in the analysis of drugs in OF. The use

of coca products in the investigation of the kinetics of cocaine and cocaine derivatives in OF and other biological matrices could reduce the ethical issues associated with the administration of drugs of abuse in clinical studies.

It is suggested that paired OF and blood samples be collected following the exposure of coca tea and/or coca products to evaluate S/P ratios (Haeckel and Hänecke 1996). Blood samples were not collected while developing the *in vivo* study because of the increase in ethical issues associated to the collection of blood samples and limitations on the logistics of sample collection and transport (exportation/importation) of samples from Colombia into the UK. The consumption of coca tea and coca products is illegal and not socially accepted in the UK which limits the ethical approval of the study if this is to be conducted in the UK. Furthermore, a Home Office import licence needs to be obtained to import the coca tea (or coca products) to the UK. Hence, further collaborations between research groups in South America and the UK is recommended for future studies as this could help reduce ethical limitations and cost. Although transportation of biological samples between countries could increase the cost of the study if samples need to be analysed in the UK.

Further studies need to be conducted in order to confirm the equivalence between the A and B samples when these have been collected simultaneously using two Concateno Certus® OF collection devices. A large number of samples should be evaluated to determine the error in the equivalence of sample concentration and whether any significant differences in drug concentration in OF are the result of inaccurate collection devices, collection procedure (position of the device in the oral cavity and/or active sample collection) or differences in the concentrations of drugs from different sections of the oral cavity.

In this thesis, the *in vitro* release of drugs from oral depots into OF and the *in vitro* permeability across oral mucosa were evaluated and conducted using separate studies, however, these two processes take place simultaneously. Therefore, more studies need to be conducted to determine if there is an increase in the release rate of cocaine in OF when cocaine has been transported from the basolateral side across the oral tissue into OF as well as being excreted from oral depots into OF.

Even though the passive transport of drugs across oral mucosa was developed using fresh porcine mucosa, the bioavailability of the tissue could not be confirmed because of limitations with the instrumentation required to analyse the samples. Evaluation of the preservation of the tissue is recommended in the future to verify the release rate obtained in this study. To achieve this, the diffusion studies should be conducted under carbogen conditions (95% O_2 + 5% CO_2) and the integrity of the oral mucosa needs to be check before and after the diffusion studies (Pather et al. 2008) using any of the methods described in Section 1.9.2.

Cell-based *in vitro* methods (Hamster check pouch cells (HPBE), epithelial cells of Madin-Darby canine kidney (MDCK), TR146 cell line and human oral keratinocytes) are also recommended to evaluate the transport of cocaine, cocaine derivatives and other drugs across the oral mucosa (Nicolazzo and Finnin 2008, Kolli and Indiran 2015, Castro et al. 2016). In particular, the EpiOral™ model offers a buccal 3D tissue model, which is widely used for the analysis of barrier function, permeability and toxicity (Castro et al. 2016). Although these methods require of more controlled conditions (cell cultured conditions), they offer the advantage of allowing the evaluation of the passive and active diffusion of drugs which could be applied for the study of drug release in OF.

The results presented in this thesis showed the potential of Raman microscopy as an alternative technique for the quantitative analysis of cocaine in porcine oral tissue and OF. It is recommended that further studies be conducted on the quantitation of cocaine in OF and oral tissue by more sensitive Raman spectrometers and/or Raman microscopy to increase the sensitivity of the method and be able to apply this technique on drug testing. Mapping of oral tissue following exposure to cocaine using Raman microscopy should be conducted to identify the position of drug depots in the tissue and evaluate whether drugs depots are homogeneity distributed or not within the oral tissue. This study could further enhance our understanding of how drugs are released from oral depots into OF. Although cocaine was the only analyte evaluated by Raman spectroscopy, this technique could be further expanded to the detection and quantitation of cocaine metabolites and other drugs of abuse such as cannabis and opioids.

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APPENDIX A – Protocol for Synthetic Oral Fluid Preparation

Table 1. Protocol for the preparation of one litre of synthetic oral fluid (Cozart Bioscience 2008)

Materials	Quantity	Supplier
De-ionised water	800 mL	Fisher Scientific
Di-sodium hydrogen orthophosphate anhydrous	2.30 g	BDH
Potassium dihydrogen orthophosphate	0.40 g	BDH
Sodium chloride	8.77 g	BDH
Bovine Serum Albumin (BSA) (1.0 % w/v)	3.00 g	Sigma - Aldrich
Glucose	0.01 g	Fisher Scientific
Mucin	0.50 g	Sigma - Aldrich
Amylase Type II - A	0.25 g	Sigma - Aldrich
Sodium azide (0.05 %)	0.50 g	BDH
5-Bromo-5-Nitro-1,3 Dioxane	0.10 g	Sigma - Aldrich
Polyvinyl Alcohol	4.50 g	Sigma - Aldrich
De-ionised water	Make up to 1000 mL	Fisher Scientific

Preparation instructions:

1. Add the above ingredients but allow the BSA to dissolve before adding additional ingredients
2. Mix on a magnetic stirrer until the PVA has dissolved

APPENDIX B – Results of peak identification using Raman spectroscopy

Table 1. Identification of functional groups obtained from Raman spectra of cocaine using the portable iRaman spectrometer equipped with 785 nm laser power.

Raman shift (cm^{-1})										Assignment
Coc[1]	S2[2]	S2+OF[3]	S2+Coc[4]	S4[5]	S4+OF[6]	S4+Coc[7]	S5[8]	S5+OF[9]	S5 + Coc[10]	Functional group / Compounds
	413	413	413		393	393				cholesterol
		530	522	517	520	522				S-S disulfide stretching from proteins
		637-689	617-687			617			613	C-C twist aromatic ring from proteins
618	689									(–C–C–) stretch
784		728	775		727	717			790	(–C–C–) stretch. Symmetric breathing of tryptophan (protein assignment)
824			887		890	860-887				Tropine ring stretch
868	891			858					895	(–C–C–) stretch
896	943	914								Aromatic ring breathing mode
998			1000			1000			1000	Phenyl aromatic ring breathing mode
1079	1093	1026-1089	1026-1093		1049	1049				$\nu(PO_2^-)$, ν(CN), ν(CC) Nucleic acids, lipids, proteins
1158	1192	1121	1121		1123	1141			1164	Amide III (β sheet structure). $N(PO_2^-)$, nucleic acids. Cellular nucleic acids, a concentred ring mode proteins, including collagen.
1273	1233	1207	1215	1127 - 1404	1221	1247			1258	CH_2 bending mode of proteins, lipids and aliphatic aminoacicds
	1346	1346	1346							
1452	1406	1450	1452		1474	1478			1445	C-C stretching. δ(C=C), phenylalanine, Oxy-Mb (haeme core)
1596	1540	1540-1589	1540-1596	1567 - 1600	1569	1571			1554	Aromatic ring (–C=C–) stretch and amide I from proteins
1713			1716			1716				Esters, C=O stretching (lipids, tropine ring, symmetric and asymmetric phenyl ring mode)

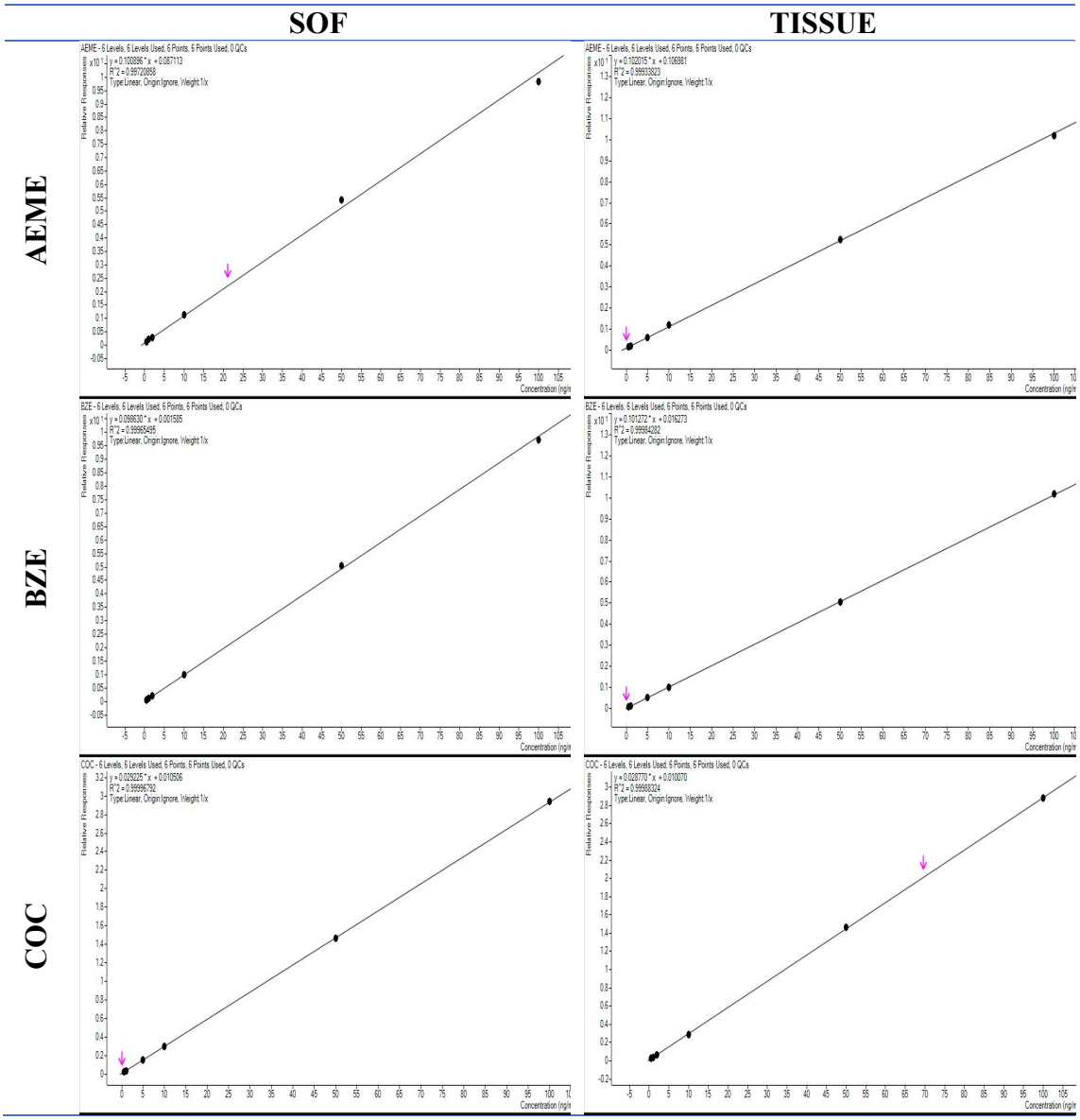
Coc: Cocaine hydrochloride (solid), S2: Substrate 2, S2+OF: Substrate 2 with OF, S2+Coc: Substrate 2 with solution of Coc in OF at 20 mg/mL, S4: Substrate 4, S4+OF: Substrate 4 with OF, S4+Coc: Substrate 4 with solution of Coc in OF at 20 mg/mL, S5: Substrate 5, S5+OF: Substrate 5 with OF, S5+Coc: Substrate 5 with solution of Coc in OF at 20 mg/mL.

Table 2. Identification of functional groups obtained from Raman spectra using the Themo Scientific DXR Raman microscope equipped with 532 nm and 785 nm laser power.

Raman shift (cm^{-1})					Assignment
Cocaine Free base	532 nm		780 nm		Functional group / Compounds
	CT[1]	ET[2]	CT[1]	ET[2]	
	747	748			Symmetric breathing of tryptophan (protein assignment), Mb (porphyrin core)
788					Piperidine ring (–C–C–) stretch
847					Tropine ring stretch
872					Pyrrolidine ring (–C–C–) stretch
896					Aromatic ring breathing mode
1003		1002		1003	Aromatic ring breathing mode from tropine
	1121	1125	1122		Amide III, $N(PO_2^-)$, nucleic acids. Cellular nucleic acids, ring mode proteins, including collagen.
	1298	1309	1294		CH_2 Lipids, protein (aliphatic amino acids)
	1439	1310-1443	1439	1340, 1450	CH_2 bending mode of proteins & lipids δ as (CH_3) δ(CH_2) of proteins. CH_2CH_3 deformation
	1580	1583			C-C stretching. δ(C=C), phenylalanine, Oxy-Mb (haeme core)
1601					Aromatic ring (–C=C–) stretch
	1638				Differences in collagen content. Amide I protein band
	1655	1644	1657	1657	(C=C) Amide I/protein amide I absorption lipid (C=C stretch)
1713	1744	1709			Esters, C=O stretching (lipids)

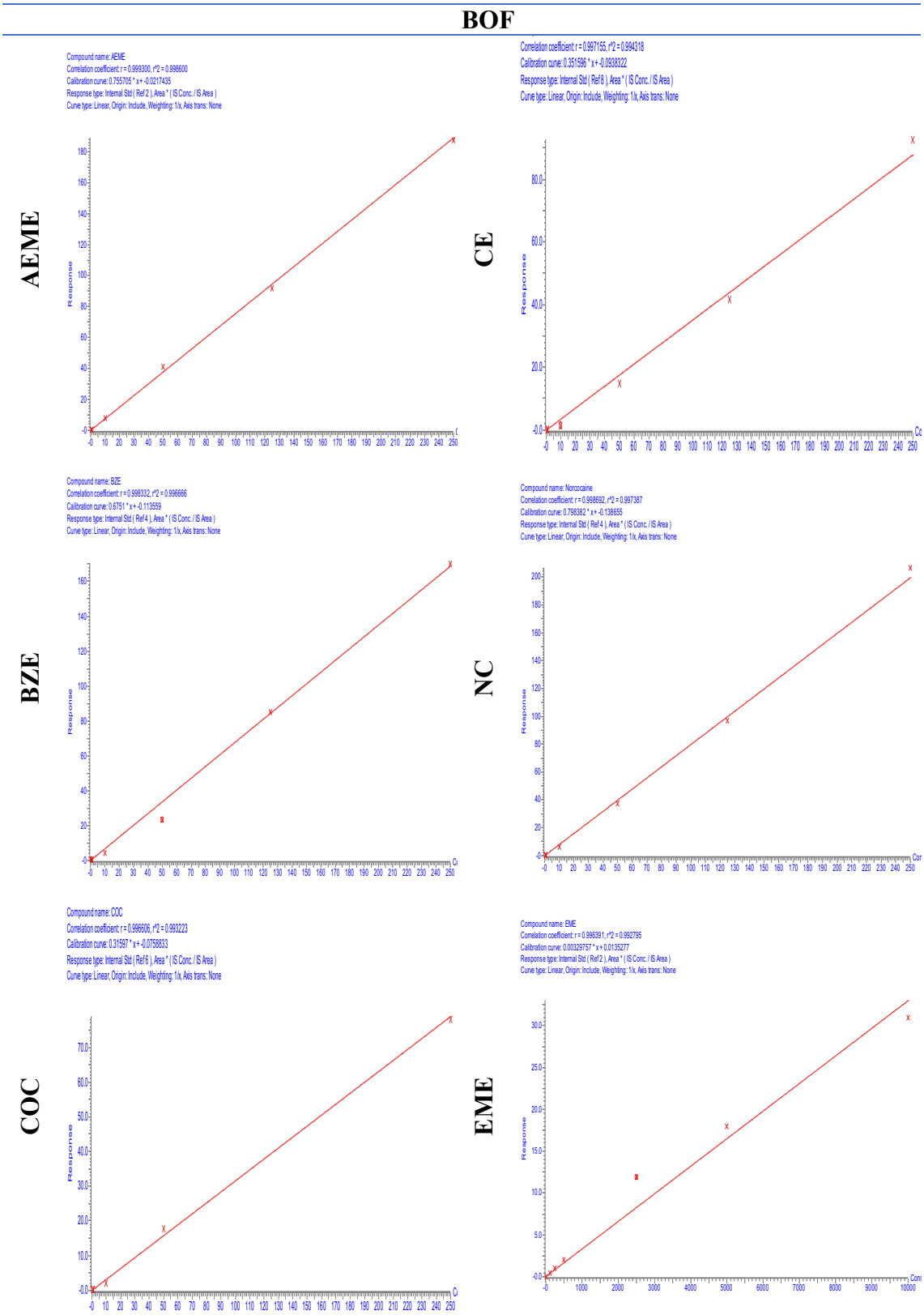
CT: Control tissue, ET: Tissue containing cocaine

APPENDIX C – Validation of LC-MS methods for the quantitation of cocaine and cocaine derivatives in synthetic oral fluid, buffered oral fluid and porcine oral mucosa



AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, SOF: Synthetic oral fluid.

Figure 1. Calibration lines obtained for cocaine and cocaine derivatives in synthetic oral fluid and homogenised porcine oral tissue.



AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, CE: Cocaethylene, EME: Ecgonine methyl ester, NC: Norcocaine, BOF: Buffered oral fluid.

Figure 2. Calibration lines obtained for cocaine and cocaine derivatives in buffered oral fluid.

APPENDIX D – Stability

Table 1. Stability data of AEME and BZE in homogenised porcine oral mucosa

		AEME						BZE					
	HOUR	LOW		MED		HIGH		LOW		MED		HIGH	
		Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD
37° C	0	4.7	0.6	23.0	1.4	82.7	2.0	4.6	0.9	19.4	0.9	77.5	0.9
	0.25	4.6	3.1	22.8	0.5	78.9	0.9	3.7	1.2	16.0	2.3	66.2	2.1
	0.5	4.5	1.3	21.7	1.3	80.4	0.5	4.2	0.9	17.7	3.3	77.0	0.4
	1	4.6	1.5	22.1	1.3	81.7	1.2	3.8	1.2	19.9	1.0	63.1	0.9
	4	4.7	1.2	21.4	1.2	79.0	5.3	4.6	1.2	18.4	0.8	68.9	1.0
	24	4.9	1.7	23.4	9.8	81.2	0.9	4.0	2.5	16.9	4.6	74.8	3.4
	DAY												
-20° C	0	4.7	1.6	23.0	1.6	82.7	1.5	4.9	4.3	19.2	1.8	77.5	1.4
	1	4.9	4.3	23.9	3.2	87.2	4.3	5.3	2.2	21.3	0.3	87.5	0.1
	4	5.2	3.4	24.9	3.8	80.7	0.5	5.1	3.1	21.7	0.2	82.4	0.7
	7	4.9	2.8	23.6	1.7	91.6	4.2	5.4	4.3	20.8	3.5	81.8	0.5
	15	5.4	3.8	23.3	2.6	93.9	2.1	5.1	1.3	19.2	1.2	82.6	3.0
	30	5.2	1.2	26.7	4.1	89.8	0.6	5.6	2.0	21.2	1.4	78.5	4.3

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, CE: Cocaethylene, EME: Ecgonine methyl ester, NC: Norcocaine.

Table 2. Stability data of cocaine in homogenised porcine oral mucosa

		COC						BZE					
	HOUR	LOW		MED		HIGH		LOW		MED		HIGH	
		Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD	Mean (ng/mL)	%RSD
37° C	0	4.3	3.8	18.8	0.9	71.8	0.1	0.2	3.6	0.3	20.8	0.6	7.9
	0.25	4.3	0.6	19.0	2.2	70.6	2.6	0.3	43.0	0.5	2.2	0.7	12.7
	0.5	4.2	0.7	18.5	0.7	71.3	0.5	0.3	6.9	0.3	9.2	0.7	1.4
	1	4.0	1.2	17.8	0.4	69.6	1.3	0.3	14.3	0.5	11.5	1.0	6.0
	4	3.9	0.1	18.1	0.8	68.2	1.2	0.3	15.3	0.4	23.0	1.3	2.3
	24	3.8	0.7	18.2	6.5	66.5	0.0	0.5	3.8	1.4	8.3	4.4	12.0
	DAY												
-20° C	0	4.3	3.7	18.8	4.3	71.8	4.2	0.2	4.3	0.1	4.5	0.0	3.2
	1	4.2	4.2	19.5	4.0	75.6	4.5	0.0	3.1	0.0	1.0	0.0	3.1
	4	4.7	2.9	20.9	0.1	75.1	1.8	0.1	3.1	0.1	3.2	0.0	3.4
	7	4.7	2.4	20.3	3.3	72.6	2.0	0.1	2.4	0.1	4.2	0.1	2.7
	15	4.4	1.1	22.1	3.9	82.6	3.3	0.2	1.7	0.1	2.4	0.1	0.6
	30	5.0	1.7	21.3	3.0	77.4	2.4	0.1	1.2	0.0	0.2	0.0	4.5

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, CE: Cocaethylene, EME: Ecgonine methyl ester, NC: Norcocaine.

Table 3. Stability data of AEME and BZE in synthetic oral fluid

	HOUR/ DAY	AEME						BZE					
		LOW		MED		HIGH		LOW		MED		HIGH	
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
37° C	0	5.7	2.1	21.8	1.9	81.0	0.8	4.8	0.9	18.3	0.9	76.4	0.9
	0.25	5.5	1.3	22.3	1.6	82.6	0.7	3.9	1.2	17.5	2.3	64.7	2.1
	0.5	5.5	1.1	22.1	0.9	80.8	0.9	4.4	0.9	15.5	3.3	77.4	0.4
	1	5.1	0.4	20.9	0.7	79.6	2.8	3.9	1.2	15.5	1.0	69.1	0.9
	4	5.3	3.1	21.0	3.3	80.6	1.1	4.1	1.2	17.0	0.8	76.9	1.0
	24	5.1	4.2	20.1	0.5	73.3	1.0	4.2	2.5	16.0	4.6	75.6	3.4
Room Temperature	0	6.5	0.5	23.7	0.5	90.5	0.5	5.9	2.2	21.9	2.2	88.4	2.2
	4	6.1	1.5	22.5	3.0	87.7	0.8	4.7	2.3	18.2	12.0	73.4	2.3
	8	6.6	3.4	21.1	6.5	87.1	11.1	3.9	2.5	15.9	5.7	56.7	2.4
	15	5.4	0.8	19.6	1.4	78.8	2.9	4.0	1.2	15.1	1.2	58.1	2.2
	30	4.4	13.4	18.1	3.1	69.0	5.5	3.5	2.1	13.6	1.5	56.7	1.2
	60	3.9	3.0	14.2	1.4	57.1	4.8	2.8	10.4	9.7	7.7	44.2	1.5
	90	2.2	4.0	8.1	3.2	32.8	2.9	1.9	2.3	7.1	1.5	28.2	3.0
Fridge	0	6.5	0.5	23.7	0.5	90.5	0.5	5.9	0.9	21.9	0.9	88.4	0.9
	4	6.2	0.8	22.2	2.5	93.3	3.8	5.5	1.2	19.8	2.3	83.7	2.1
	8	6.5	3.1	21.7	4.1	85.8	2.6	6.0	0.9	18.3	3.3	75.6	0.4
	15	5.8	0.9	21.2	1.2	86.2	1.5	5.3	1.2	19.6	1.0	80.8	0.9
	30	6.0	0.9	20.8	2.4	82.9	2.6	5.1	1.2	19.4	0.8	80.4	1.0
	60	5.7	1.4	19.9	0.9	79.2	0.8	4.7	2.5	16.1	4.6	69.7	3.4
	90	5.0	2.0	16.5	14.5	74.6	1.4	4.4	0.9	14.0	0.7	72.9	1.1
-20° C	0	6.5	2.2	23.7	2.2	90.5	2.2	5.6	1.8	21.4	1.8	87.3	1.8
	4	6.2	4.0	22.6	2.6	84.8	1.7	5.2	1.6	19.4	1.1	81.1	1.5
	8	6.7	1.3	22.3	2.1	89.3	1.3	4.5	1.4	17.8	0.7	72.1	1.1
	15	5.8	6.7	21.1	2.8	82.4	1.1	5.2	0.8	19.5	0.9	81.5	0.8
	30	5.3	0.9	21.0	4.8	83.7	1.4	5.1	1.9	19.4	0.8	80.3	1.5
	60	5.6	2.3	19.8	1.7	80.5	5.1	4.5	3.1	18.0	2.8	69.7	4.9
	90	5.8	1.2	21.2	4.9	83.9	0.7	5.3	3.4	20.4	2.9	78.4	2.6

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, CE: Cocaethylene, EME: Ecgonine methyl ester, NC: Norcocaine.

Table 4. Stability data of cocaine in synthetic oral fluid

	HOUR/DAY	COC						BZE					
		LOW		MED		HIGH		LOW		MED		HIGH	
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
37° C	0	4.4	0.1	17.8	0.2	69.2	1.2	0.3	1.9	0.5	1.3	1.4	1.0
	0.25	4.3	0.1	17.4	1.8	69.2	0.9	0.3	1.4	0.7	1.6	2.3	0.3
	0.5	4.3	2.0	17.3	0.7	70.6	1.0	0.4	5.3	0.9	1.1	3.1	2.0
	1	4.0	1.9	16.8	1.3	67.5	1.6	0.5	0.2	1.4	1.3	5.3	1.7
	4	3.7	1.7	14.9	2.6	59.5	0.4	0.8	0.4	3.0	1.8	12.4	0.8
	24	2.5	6.0	7.5	0.7	25.4	1.4	2.5	1.2	10.1	0.2	39.5	1.1
Room Temperature	0	6.1	0.8	21.5	0.8	88.5	0.8	0.3	2.1	0.5	9.8	1.1	6.7
	4	4.9	0.6	17.1	1.5	71.1	1.6	1.2	5.8	3.7	3.8	5.7	5.1
	8	3.6	11.0	6.8	17.7	24.7	22.2	2.0	1.3	6.0	6.3	6.6	7.8
	15	2.8	0.4	9.8	2.7	38.6	0.9	1.9	4.3	6.7	9.9	32.6	3.0
	30	0.8	2.4	2.3	0.4	9.8	0.3	2.5	2.9	8.3	2.1	45.4	8.8
	60	0.8	13.2	1.3	5.9	4.1	5.7	3.1	1.2	12.2	1.2	39.5	0.5
	90	0.6	2.8	1.6	6.5	4.4	6.1	4.0	5.4	14.7	2.0	47.3	1.9
Fridge	0	6.1	1.1	21.5	1.1	88.5	1.1	0.3	6.8	0.5	6.8	1.1	8.8
	4	5.6	1.0	20.1	1.5	82.7	2.1	0.0	3.1	1.5	9.1	4.9	9.1
	8	4.9	8.8	18.1	6.1	75.6	1.3	0.7	1.3	0.5	5.7	4.6	0.8
	15	5.7	2.1	19.8	0.4	82.2	1.6	0.9	6.7	2.7	0.4	11.9	7.6
	30	3.8	0.7	13.5	0.7	56.5	0.5	1.8	0.4	4.9	6.8	19.3	3.2
	60	3.4	17.8	11.9	2.4	50.3	3.6	1.7	4.6	4.4	5.4	27.5	0.2
	90	2.0	12.8	5.9	0.8	25.2	1.3	3.0	8.7	11.6	8.3	43.9	2.5
-20° C	0	6.1	2.2	21.5	2.2	88.5	2.2	0.3	9.2	0.5	4.9	1.1	9.2
	4	5.6	1.6	20.0	0.7	83.5	1.8	0.4	7.2	0.5	6.5	1.2	6.7
	8	6.0	0.8	19.7	0.6	78.9	1.7	0.4	1.2	0.5	3.3	1.1	3.4
	15	6.4	0.4	23.5	0.8	96.9	1.8	0.4	5.4	0.7	5.8	1.3	2.7
	30	5.4	2.6	20.0	0.9	82.6	1.6	0.4	7.3	0.5	2.6	1.1	7.5
	60	6.2	9.6	22.3	1.2	93.0	4.6	0.4	8.4	0.5	9.0	1.3	1.4
	90	6.7	21.5	24.4	0.8	101.0	0.3	0.3	6.9	0.4	3.2	1.4	7.8

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, CE: Cocaethylene, EME: Ecgonine methyl ester, NC: Norcocaine.

Table 5. Stability data of AEME, EME, BZE, CE and NC in buffered oral fluid stored at room temperature

		LOW		MED		HIGH	
N = 3	**DAY**	**Mean (ng/mL)**	**%RSD**	**Mean (ng/mL)**	**%RSD**	**Mean (ng/mL)**	**%RSD**
AEME	0	4.4	3.4	47.5	3.4	189.9	3.4
	1	5.3	2.1	49.4	1.5	192.4	1.6
	4	4.7	4.1	50.0	5.0	198.5	2.5
	8	4.7	4.9	47.9	2.9	193.0	2.6
	11	5.1	14.8	47.9	8.9	205.7	1.1
	15	6.1	2.8	54.6	5.7	211.6	7.4
	30	5.4	7.1	51.4	2.2	197.0	4.7
	60	5.6	10.1	47.7	1.6	182.7	3.8
EME	0	81.3	10.0	1037.9	10.0	3870.6	10.0
	1	71.5	5.1	859.7	15.1	2612.8	3.6
	4	14.9	7.9	165.0	10.2	842.1	13.4
	8	49.1	18.2	634.2	17.2	2303.8	15.7
	11	40.0	22.8	470.7	16.1	2251.6	2.1
	15	34.6	37.7	432.4	8.6	2214.0	32.5
	30	4.1	173.2	66.5	54.5	248.0	43.4
	60	51.3	29.7	208.5	35.4	1720.8	13.9
BZE	0	4.1	4.2	45.2	4.2	170.5	4.2
	1	3.5	11.7	41.7	2.6	156.7	1.6
	4	4.6	1.5	48.8	4.6	185.8	2.9
	8	3.3	1.9	35.8	2.9	149.6	3.4
	11	3.7	13.5	40.5	2.7	167.1	0.6
	15	3.4	5.9	36.5	3.3	162.6	1.8
	30	3.4	14.8	45.7	1.8	203.3	3.4
	60	3.0	22.0	35.5	7.7	144.0	12.8
CE	0	4.9	3.1	53.0	3.1	182.1	3.1
	1	4.0	2.3	52.0	2.2	171.7	1.5
	4	4.8	3.6	51.6	3.9	153.2	1.7
	8	4.0	5.6	46.9	1.7	185.8	1.2
	11	3.8	14.4	39.6	9.5	161.8	2.5
	15	2.3	2.5	27.8	2.9	135.8	2.7
	30	2.9	10.4	43.5	3.5	137.5	2.7
	60	1.9	5.7	25.1	2.1	115.5	4.1
NC	0	3.3	13.2	34.7	1.0	154.1	1.0
	1	2.7	0.4	26.1	2.1	123.7	2.7
	4	3.7	3.1	38.7	6.4	157.3	6.6
	8	2.9	4.9	30.6	3.8	136.6	4.9
	11	3.2	13.6	31.1	7.6	141.5	3.3
	15	2.9	4.0	29.5	6.9	124.3	8.8
	30	2.7	10.5	37.5	12.7	157.8	7.3
	60	2.9	4.7	30.8	3.7	153.0	16.0

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, CE: Cocaethylene, EME: Ecgonine methyl ester, NC: Norcocaine.

Table 6. Stability data of AEME, EME, BZE, CE and NC in buffered oral fluid stored in the fridge

		LOW		MED		HIGH	
N = 3	**DAY**	**Mean (ng/mL)**	**%RSD**	**Mean (ng/mL)**	**%RSD**	**Mean (ng/mL)**	**%RSD**
AEME	0	4.4	3.1	47.5	3.1	189.9	3.1
	1	5.3	6.8	48.5	11.3	193.2	2.5
	4	4.7	1.1	49.7	1.4	200.5	0.1
	8	4.5	8.8	46.6	6.0	189.7	5.4
	15	5.0	10.4	53.8	6.5	209.4	12.4
	30	5.1	5.4	49.0	0.7	199.3	3.4
	60	4.8	7.9	49.8	2.7	189.2	9.1
EME	0	81.3	4.0	1037.9	4.0	3870.6	4.0
	1	78.1	5.7	700.3	12.6	2741.7	33.2
	4	16.8	6.4	181.9	7.3	748.4	9.7
	8	60.8	10.6	693.7	6.9	3063.8	19.6
	15	45.4	11.4	493.9	10.0	2302.5	65.6
	30	7.0	129.2	90.0	12.6	363.9	126.3
	60	39.4	88.5	13.0	22.9	7.8	34.9
BZE	0	4.1	13.8	45.2	4.0	170.5	13.8
	1	3.4	7.8	41.8	12.6	159.0	6.4
	4	4.5	0.5	48.5	7.3	184.6	3.1
	8	3.3	2.5	36.8	6.9	140.6	2.0
	15	3.5	14.5	35.9	10.0	169.0	17.0
	30	3.1	11.6	41.4	12.6	198.4	12.8
	60	2.6	44.5	39.3	22.9	182.0	10.8
CE	0	4.9	1.8	53.0	1.8	182.1	1.8
	1	4.2	7.3	51.7	10.8	156.0	12.9
	4	5.0	3.3	54.0	1.5	148.5	4.8
	8	4.7	8.0	52.2	4.0	211.8	4.7
	15	4.0	11.6	42.4	7.6	151.2	4.1
	30	4.3	7.9	55.5	2.1	152.9	1.9
	60	2.5	57.6	41.7	3.3	176.8	6.3
NC	0	3.3	3.4	34.7	3.4	154.1	3.4
	1	2.7	6.2	32.5	18.8	135.6	16.8
	4	3.7	6.4	39.2	7.1	163.9	6.9
	8	3.0	9.3	29.9	4.8	130.0	6.6
	15	3.0	11.1	34.6	9.1	146.1	17.5
	30	3.6	16.2	40.4	6.2	170.2	9.3
	60	2.6	2.1	29.5	14.0	130.1	8.6

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, CE: Cocaethylene, EME: Ecgonine methyl ester, NC: Norcocaine.

Table 7. Stability data of AEME, EME, BZE, CE and NC in buffered oral fluid stored at -20 °C

		LOW		MED		HIGH	
N = 3	**DAY**	**Mean (ng/mL)**	**%RSD**	**Mean (ng/mL)**	**%RSD**	**Mean (ng/mL)**	**%RSD**
AEME	0	4.4	6.5	47.5	6.5	189.9	6.5
	1	5.2	2.0	49.7	2.8	191.2	2.0
	4	4.7	0.5	48.7	2.5	198.0	2.0
	8	4.4	5.3	45.9	3.5	195.3	8.1
	15	4.9	2.9	43.7	11.3	204.9	3.0
	30	4.3	0.6	46.4	1.9	186.6	6.8
	60	4.0	8.2	40.4	5.0	184.3	6.7
EME	0	81.3	8.5	1037.9	8.5	3870.6	8.5
	1	61.8	5.0	818.8	2.2	2518.6	46.8
	4	14.5	14.0	188.6	14.7	918.9	11.8
	8	69.2	1.4	648.0	4.8	3054.1	19.0
	15	40.2	20.8	189.1	48.1	2144.2	40.8
	30	7.9	5.7	99.1	5.0	432.5	129.4
	60	20.9	35.6	15.3	20.6	411.5	26.6
BZE	0	4.1	15.1	45.2	15.1	170.5	15.1
	1	3.6	1.7	44.6	14.4	159.0	5.7
	4	4.5	2.1	48.6	1.8	180.4	2.6
	8	3.3	0.6	36.3	1.8	140.3	5.5
	15	3.3	2.5	38.0	7.5	166.1	4.1
	30	3.4	1.0	38.2	2.7	181.0	7.2
	60	2.8	13.2	33.5	5.8	152.0	3.5
CE	0	4.9	6.8	53.0	6.8	182.1	6.8
	1	4.0	3.3	52.6	2.0	172.6	10.5
	4	5.0	0.6	54.4	1.6	145.8	1.2
	8	5.0	1.1	55.2	3.6	226.7	9.7
	15	4.3	4.7	44.9	17.5	153.9	4.6
	30	5.6	2.3	63.7	1.9	160.2	0.4
	60	4.7	65.4	62.7	3.1	209.6	6.8
NC	0	3.3	11.1	34.7	11.1	154.1	11.1
	1	3.0	6.7	31.1	17.7	133.1	7.8
	4	3.7	6.5	37.9	2.8	156.0	5.7
	8	2.9	3.7	30.1	3.5	133.8	9.2
	15	3.0	6.1	26.5	6.2	147.7	9.7
	30	3.5	2.5	39.6	4.8	154.8	1.5
	60	2.8	4.6	30.2	9.7	132.8	12.0

AEME: Anhydroecgonine methyl ester, BZE: Benzoylecgonine, COC: Cocaine, CE: Cocaethylene, EME: Ecgonine methyl ester, NC: Norcocaine.

APPENDIX E

Bournemouth University

Research Ethics Checklist

Reference Id	12788
Status	Approved
Date Approved	06/09/2016

Researcher Details

Name	Aida Merchan Otalora
Faculty	Faculty of Science & Technology
Status	Postgraduate Research (MRes, MPhil, PhD, DProf, DEng)
Course	Postgraduate Research - FST
Have you received external funding to support this research project?	Yes
RED ID	8330
Funding Body	Alere Toxicology, Abingdon
Please list any persons or institutions that you will be conducting joint research with, both internal to BU as well as external collaborators.	Professor David Osselton, Head of Department of Archaeology, Anthropology and Forensic science, Bournemouth University. Dr Sulaf Assi, Lecture in Forensic Science, Bournemouth University, Dr Claire George, Alere Toxicology, Abingdon, UK. Aida Merchan, PhD student in Forensic Toxicology, Bournemouth University, Jorge Ariel Martinez, Head of department of Pharmacy, Universidad Nacional de Colombia – Bogota.

Project Details

Title	Evaluation of the concentration of cocaine derivatives from collected oral fluid samples following consumption of coca tea.

Proposed Start Date of Data Collection	10/10/2016
Proposed End Date of Project	08/12/2016
Original Supervisor	David Osselton
Approver	David Osselton

Summary - no more than 500 words (including detail on background methodology, sample, outcomes, etc.)

Procedures commonly used to collect blood or urine samples in workplace or police environments are regarded as invasive either because the subject is subjected to having blood collected via a syringe and needle or urine collected under “observed” collection conditions. During recent years research has demonstrated that drugs are excreted via the saliva into the mouth hence the collection and analysis of saliva (oral fluid) is growing in popularity. The initial concept of drug entry into saliva and oral fluid (OF) was based on a philosophy that that drugs present in the blood could pass through the cell membranes of the salivary glands across a concentration gradient, the extent of which would be determined by the lipid solubility of the drug and its pKa value. Whilst this theory holds for many medicinal drugs consumed in tablet formulations Osselton et al (2001) demonstrated that for heroin and cocaine, which are commonly consumed via nasal insufflation or smoking, the detection times are significantly longer that would be expected using pharmacokinetic models. This lead to the proposition that cocaine and heroin can form depots in the mouth tissues following exposure and are subsequently released from the tissue over time. If drugs are accumulated in oral tissue depots and are subsequently released over time, this has significant potential implications when oral fluid is used for monitoring drug use by an individual. Reichardt (PhD Thesis Bournemouth University) demonstrated that following exposure of buccal tissue to coca tea, cocaine derivatives are released from buccal tissues over a period of several hours. The study also revealed a number of unexplained artefacts relating to the detection of cocaine derivatives in oral fluid collected after the consumption of coca tea. This study aims to further our understanding of cocaine absorption and elimination from oral/buccal tissues by analysing the oral exudate collected from volunteers following the consumption of coca tea. The study will be undertaken in collaboration with the National University of Colombia where consumption of coca tea is legal and regular social custom The study aims to engage 30 adult volunteers to consume a standard cup of tea and subsequently collect oral fluid samples at timed intervals over a period of 4 hours using a commercial oral fluid collection device. It is proposed to collect oral fluid samples at 10, 20, 30, 60, 120, 180 and 260 minutes post consumption. Oral fluid samples will be analysed at Alere Toxicology, Abingdon, UK using a validated LC-MS method.Coca tea contains small quantities of cocaine derivatives such that at high altitude it eases breathing and reduces the symptoms of altitude sickness. Coca tea does not produce any form of “high” or intoxication and is significantly less likely to affect an individual than the social use of alcohol.

External Ethics Review

Does your research require external review through the NHS National Research Ethics Service (NRES) or through another external Ethics Committee?	**No**

Research Literature

Is your research solely literature based?	**No**

Human Participants

Will your research project involve interaction with human participants as primary sources of data (e.g. interview, observation, original survey)?	Yes
Does your research specifically involve participants who are considered vulnerable (i.e. children, those with cognitive impairment, those in unequal relationships—such as your own students, prison inmates, etc.)?	No
Does the study involve participants age 16 or over who are unable to give informed consent (i.e. people with learning disabilities)? NOTE: All research that falls under the auspices of the Mental Capacity Act 2005 must be reviewed by NHS NRES.	No
Will the study require the co-operation of a gatekeeper for initial access to the groups or individuals to be recruited? (i.e. students at school, members of self-help group, residents of Nursing home?)	No
Will it be necessary for participants to take part in your study without their knowledge and consent at the time (i.e. covert observation of people in non-public places)?	No
Will the study involve discussion of sensitive topics (i.e. sexual activity, drug use, criminal activity)?	Yes
Are drugs, placebos or other substances (i.e. food substances, vitamins) to be administered to the study participants or will the study involve invasive, intrusive or potentially harmful procedures of any kind?	Yes

Please explain why your research project does not require ethical review by a NHS REC.
None of the volunteers participating in this study will be patients or service users within the services for which the UK Health Departments are responsible.Only volunteers who participate with informed consent will be permitted to enter the study. The experimental protocol and reasoning behind the study will be explained in full, samples identity will be anonymised and volunteers will be required to sign a consent form agreeing participation in the study. The study will be carried out in Colombia where coca tea is widely consumed and where its use is both legal and socially acceptable. The quantity of cocaine present in coca tea is very low and the oral route of administration of this beverage does not lead to harmful or significant side effects. The tea will be prepared using commercial coca tea bags (Nasa esh's) purchased from the Nasa community (indigenous community). This will simply be following a local custom of drinking coca tea.

Will tissue samples (including blood) be obtained from participants? Note: If the answer to this question is 'yes' you will need to be aware of obligations under the Human Tissue Act 2004.	No
Could your research induce psychological stress or anxiety, cause harm or have negative consequences for the participant or researcher (beyond the risks encountered in normal life)?	No
Will your research involve prolonged or repetitive testing?	Yes
Will the research involve the collection of audio materials?	No
Will your research involve the collection of photographic or video materials?	No
Will financial or other inducements (other than reasonable expenses and compensation for time) be offered to participants?	No

Please explain below why your research project involves the above mentioned criteria (be sure to explain why the sensitive criterion is essential to your project's success). Give a summary of the ethical issues and any action that will be taken to address these. Explain how you will obtain informed consent (and from whom) and how you will inform the participant(s) about the research project (i.e. participant information sheet). A sample consent form and participant information sheet can be found on the Research Ethics website.
The sensitive question will relate to asking whether the volunteer has used drugs for either recreational or medical use within a period of 72 hours prior to the consumption of coca tea. This will eliminate subjects who may give false positives. Questions relating use of medicines will help to eliminate any subjects undergoing medical treatment. Only healthy subjects will be allowed to participate in the study and patients undergoing any form of medical treatment or suffering from any condition that requires the use of medication will be excluded. The research involves collecting eight oral fluid samples over a period of 4 hours. This may be regarded as repetitive testing?

Final Review

Will you have access to personal data that allows you to identify individuals OR access to confidential corporate or company data (that is not covered by confidentiality terms within an agreement or by a separate confidentiality	No
Will your research involve experimentation on any of the following: animals, animal tissue, genetically modified organisms?	No
Will your research take place outside the UK (including any and all stages of research: collection, storage, analysis, etc.)?	Yes
Does the country in which you are conducting research require that you obtain internal ethical approval?	No
Could conflicts of interest arise between the source of funding and the potential outcomes of the research?	No

Please use the below text box to highlight any other ethical concerns or risks that may arise during your research that have not been covered in this form.
N/A. We are undertaking the sample collection in Colombia because the consumption of coca tea is widely practised social custom.

Volunteer Information Form for Collection of Oral Fluid

Title of Project: Evaluation of the concentration of cocaine and other metabolites from collected oral fluid samples following consumption of coca tea.

Principal Investigators:
Aida Merchan, PhD student in Forensic Toxicology, Bournemouth University.
Professor David Osselton, Head of Department of Archaeology, Anthropology and Forensic science, Bournemouth University.
Dr Sulaf Assi, Lecture in Forensic Science, Bournemouth University.
Dr Claire George, Alere Toxicology, Abingdon, UK.
Dr Jorge Ariel Martinez, Lecturer at the Department of Pharmacy, Universidad Nacional de Colombia – Bogota.

You are being invited to participate in a research study. Before you decide whether you want to participate in this study, it is important that you understand why the research is being undertaken and what it will involve. Please read the following information carefully and discuss it with others if you wish. Do not hesitate to ask if anything is unclear or if you have any questions or concerns.

1. Purpose of the Research

Detection of abused drugs is a major concern for regulatory agencies worldwide. Workplace regulations and Misuse of Drugs Regulations control the production, supply or use of abused substances such as cocaine. Oral fluid drug testing has been implemented in several countries for the detection and/or analysis of drugs of abuse. Advantages in the procedure of oral fluid sample collection have positioned oral fluid as one of the most promising drug testing techniques for use in the future. There are however a number of factors related to drug elimination from the body via the saliva and oral fluid that we still do not fully understand. This study is aimed at providing information that will help us better understand how drugs enter into oral fluid.

Coca tea is produced from the leaves of the coca bush which has been planted and consumed for centuries in countries such as Colombia, Perú and Bolivia in South America. Consumption of coca leaves in these countries is both legal and socially acceptable. It is known that after the consumption of coca tea/leaves, cocaine and some breakdown products of cocaine can be detected in oral fluid.

As part of this study volunteers will be invited to drink a cup (200 mL) of coca tea (~1 mg cocaine) or swill coca tea around their mouths then spit it out without swallowing it. An oral fluid sample will be collected prior to administration of coca tea and consecutive samples of oral fluid from the inside of the cheek will be taken following administration. These samples will be collected for a period of approximately 4 hours.

A commercial oral fluid collection device (The Alere™ Certus device) will be used for collection of oral fluid. Figure 1 shows an image of the collection device. This process is non-invasive and will involve a small absorbent pad being kept in the mouth until the detector turns blue indicating a sufficient volume has been collected. The pad is then inserted into a vial containing buffer. Samples will then be stored and analysed for cocaine and its chemical breakdown products.

Figure 1. Alere™ Concateno Certus oral fluid device.

You will be requested to fill in a questionnaire providing details of age, sex and whether you are taking any drugs or medications. The questionnaire will have your assigned identity code. The information you provide and all information provided will be anonymised and confidential No impairment is caused by the consumption of coca tea/leaves.

By signing this consent it means that you have not consumed coca tea, cocaine or "crack", within the three days previous to this study. Also, that you do not have any known allergies to coca tea.

2. Discomforts and Risks

One risk of giving samples for research may be the release of your name that could be linked to the stored samples and/or the results of the tests run on the samples. To prevent this, you will be given a unique identification code. Only the research team will know the code from your samples. Only authorised members of the research team will have access to your assigned code.

There are no reports of any significant side effects after the consumption of coca tea, however in South American mountains it is widely common to drink infusions or chew coca leaves to relieve the symptoms of Altitude Mountain Sickness.

3. Statement of Confidentiality

3.1 Privacy and confidentiality measures

- Each sample will be given a unique code for which the samples will hereby be known.
- Samples are going to be stored and analysed at Alere Toxicology in the UK and interpretation of results will take place at Bournemouth University - UK. The information provided throughout this study i.e. (sex, health, drug use) will not identify you by name.
- The results of this research study may be presented at scientific/medical meetings or in scientific/medical publications. The identity of participants will not be disclosed in those presentations.
- If you agree, samples and results will be stored along with information about yourself obtained in the course of this research study (age, sex, health, drug/medication use). The information stored will not identify you by name. In the event of any publication or presentation resulting from the research, no identifiable information will be shared.
- We will keep participation in this research study confidential to the extent permitted by law. However, it is possible that other people may become aware of study participation. For example, the following people/groups may inspect and copy records pertaining to this research: The Bournemouth University Institutional Ethics Review Board (a committee that reviews and approves research studies).

- The possibility of identification is very low and every effort will be made to keep your personal information in the research record private and confidential but absolute confidentiality cannot be guaranteed. Participants will receive a signed and dated copy of this consent form for your records.

4. Costs for Participation

4.1 Costs

There are NO costs for participation in this research

4.2 Rights:

Signing this form will lose no legal rights.

4.3 Compensation

A small compensation of £10 (equivalent to approximately 40000 Colombian pesos) will be given to each participant for participation in this research study. Payment is due to be made in form of vouchers for local retailers.

5. Voluntary Participation / Withdrawal:

Participation in this research study is entirely voluntary. If you choose not to take part, you have the right to stop at any time. You can withdraw up to the point of anonymisation of data. You are free to withdraw without giving reason and without there being any negative consequences.

6. Contact Information for Questions or Concerns

You have the right to ask any questions you may have about this research. If you have questions or concerns about this research please ask the researcher Aida Merchan who is conducting the experiment at amerchanotalora@bournemouth.ac.uk or contact Professor David Osselton, Head of the Department of Archaeology, Anthropology and Forensic science, Bournemouth University, UK at dosselton@bournemouth.ac.uk.
In case of complaints, please contact Professor Christine Maggs, Executive Dean for the Faculty of Science & Technology at cmaggs@bournemouth.ac.uk

Bournemouth University

Participant Agreement Form

Title of project: Evaluation of the concentration of cocaine derivatives from collected oral fluid samples following consumption of coca tea.

- **Aida Merchan, PhD student in Forensic Toxicology, Bournemouth University.**
- **Professor David Osselton, Head of Department of Archaeology, Anthropology and Forensic science, Bournemouth University.**
- **Dr Sulaf Assi, Lecturer in Forensic Science, Bournemouth University.**
- **Dr Jorge Ariel Martinez, Lecture at the Department of Pharmacy, Universidad Nacional de Colombia – Bogotá**

Hereafter you will find a number of statements that you need to read through. Once you have read them please mark the statements that you agree with.

	Please Initial or Tick Here
I have read and understood the participant information sheet for the above research project.	
I confirm that I have had the opportunity to ask questions.	
I understand that my participation is voluntary.	
I understand that I am free to withdraw up to the point where the samples have been collected and become anonymous, so my identity cannot be determined.	
During the collection of samples, I am free to withdraw without giving reason and without there being any negative consequences.	
Should I not wish to answer any particular question(s) or give a sample, I am free to decline.	
I give permission for members of the research team to have access to my anonymised samples. I understand that my name will not be linked with the research materials and I will not be identified or identifiable in the outputs that result from the research.	
I agree to take part in the above research project.	

_____ _____ _____

Name of Participant Date Signature

_____ _____ _____

Name of Researcher Date Signature

This form should be signed and dated by all parties after the participant receives a copy of the participant information sheet and any other written information provided to the participants. A copy of the signed and dated participant agreement form should be kept with the project's main documents which must be kept in a secure location.

ETHICAL APPROVAL – NATIONAL UNIVERSITY OF COLOMBIA

UNIVERSIDAD NACIONAL DE COLOMBIA
SEDE BOGOTÁ

COMITÉ DE ÉTICA **FACULTAD DE CIENCIAS**

Bogotá, febrero 6 de 2017

Profesor
José Ariel Martínez Ramírez
Departamento de Farmacia

Respetado Profesor:

Atentamente le comunico que el Comité de Ética de la Facultad de Ciencias, en reunión realizada el día 6 de febrero de 2017 (Acta 01-2017), evaluó aspectos éticos del proyecto presentado por usted. Como resultado de esta revisión, el Comité considera que el proyecto ***cumple*** con los aspectos éticos básicos. Para los fines pertinentes, se transcriben las observaciones y el concepto final.

Proyecto: Evaluation of the concentration of cocaine and other metabolites from collected oral fluid samples following consumption of coca tea.
Proyecto conjunto entre Universidad Nacional de Colombia y Bournemouth University – UK.

Responsables: Jorge Ariel Martínez Ramírez (Investigador Principal, Universidad Nacional de Colombia, Facultad de Ciencias, Departamento de Farmacia). Coinvestigadores (Facultad de Ciencias y Tecnología – Departamento de Arqueología - Antropología y Ciencias Forenses de la "Bournemouth University – UK"): Aída Merchán, David Osselton y Sulaf Assi.

Observaciones:
Para esta investigación se colectarán, mediante un dispositivo comercialmente disponible, muestras de fluido oral de voluntarios sanos a los cuales se les suministra un té de coca. Se analizará la cinética de cocaína y sus metabolitos en fluido oral, como método alternativo al análisis tradicional en sangre y orina. Los participantes donantes de fluidos biológicos deberán firmar un formato de consentimiento informado y confidencialidad. Se hace énfasis en la necesidad de contar con las autorizaciones pertinentes para el envío de muestras biológicas al exterior.

Concepto: Avalado.

LUIS FERNANDO OSPINA G.
Coordinador Comité de Ética

5

Carrera 30 No. 45-03, **FACULTAD DE CIENCIAS**, Edificio 450, 4º piso, Oficina 400
Conmutador: (57-1) 316 5000 Ext. 14666
Correo electrónico: lfospinag@unal.edu.co / Bogotá, Colombia, Sur América

TRANSLATION: ETHICAL APPROVAL – NATIONAL UNIVERSITY OF COLOMBIA

Bogota, 6th February 2017

Professor
Jorge Ariel Martinez Ramirez
Department of Pharmacy

Dear professor:

This is to communicate that the Ethics Committee from the Faculty of Science, evaluated the ethical aspects of the project presented for you on the meeting that took place on the 6th of February 2017 (Acta 01-2017). As a result of this revision, the committee considered that the project complies with the basic ethical aspects. For the relevant purpose, the observations and the final concept are transcribed.

Project: Evaluation of the concentration of cocaine and other metabolites from collected oral fluid samples following consumption of coca tea.
A joined project between the National University of Colombia and Bournemouth University – UK.

Responsible: Jorge Ariel Martinez (Principal Investigator, National University of Colombia, Faculty of Science, Pharmacy Department). Coinvestigators (Faculty of Science and Technology – Archaeology, Anthropology and Forensic Science, Bournemouth University – UK): Aida Merchan, David Osselton and Sulaf Assi.

Observations:
For this research there will be a collection of oral fluid samples from healthy volunteer which have consumed coca tea, using a commercial device. The kinetic of cocaine and its metabolites in oral fluid will be analysed as an alternative analysis of blood and urine. Participants who donate biological fluids will have to sign an informed consent. Emphasises should be given to the need of having the required authorisations for sending biological samples abroad.

Concept: Approved

Luis Fernando Ospina G.
Coordinator Ethics Committee

APPENDIX F – Coca tea data

Table 1. AEME concentration in oral fluid for each participant measured after the ingestion or swirling of a cup of coca tea.

	Concentration Group A (ng/mL) - Ingested coca tea								Concentration Group B (ng/mL) - Swirl coca tea															
	Collection time (min)								Collection time - Right side of mouth (min)								Collection time - Left side of mouth (min)							
Participant	0	10	20	30	60	120	180	240	0	10	20	30	60	120	180	240	0	10	20	30	60	120	180	240
P1	0	167	65	38	3	0	0	0	0	418	205	67	15	0	0	0	0	514	172	73	9	0	0	0
P2	0	176	79	29	3	0	0	0	0	396	170	76	14	0	0	0	0	471	216	110	14	0	0	0
P3	0	165	36	22	1	0	0	0	0	534	408	183	35	6	0	0	0	554	335	198	46	4	0	0
P4	0	367	177	111	8	0	0	0	0	615	255	96	16	0	0	0	0	440	167	114	12	0	0	0
P5	0	347	163	60	13	1	0	0	0	130	42	16	2	0	0	0	0	121	20	0	0	0	0	0
P6	0	67	23	9	0	0	0	0	0	1275	603	340	102	2	0	0	0	1206	665	549	119	2	0	0
P7	0	655	431	193	46	0	0	0	0	524	169	138	25	12	2	0	0	459	220	135	38	9	3	0
P8	0	253	58	37	1	0	0	0	0	635	235	105	11	0	0	0	0	504	160	80	5	0	0	0
P9	0	141	58	21	4	0	0	0	0	353	167	105	21	0	0	0	0	395	228	81	15	0	0	0
P10	0	275	49	13	0	0	0	0	0	997	314	165	24	0	0	0	0	841	285	213	21	0	0	0
P11	0	37	19	7	0	0	0	0	0	271	101	33	3	0	0	0	0	203	59	20	1	0	0	0
P12	0	132	50	12	0	0	0	0	0	237	178	107	6	0	0	0	0	181	137	78	7	0	0	0
P13	0	58	12	3	0	0	0	0	0	845	551	223	16	1	0	0	0	857	429	210	48	1	0	0
P14	0	221	110	74	8	0	0	0	0	449	377	120	13	0	0	0	0	588	370	188	22	0	0	0
P15	0	44	54	15	1	0	0	0	0	668	354	155	21	0	0	0	0	525	286	121	14	0	0	0
Mean	0	207	92	43	6	0	0	0	0	556	275	128	21	2	0	0	0	524	250	145	25	1	0	0
Median	0	167	58	22	1	0	0	0	0	524	235	107	16	0	0	0	0	504	220	114	14	0	0	0
SD	0	161	105	51	12	0	0	0	0	302	159	80	24	3	0	0	0	280	159	129	30	3	1	0
Min	0	37	12	3	0	0	0	0	0	130	42	16	2	0	0	0	0	121	20	0	0	0	0	0
Max	0	655	431	193	46	1	0	0	0	1275	603	340	102	12	2	0	0	1206	665	549	119	9	3	0

Group A: Participants ingested one cup of coca tea (data collected using one collection device at each time point). Group B: Participants swirled a cup of coca tea (data collected using two collection devices at each time point). A: OF sample collected from the right side of the mouth. B: OF sample collected from the left side of the mouth. SD: Standard deviation. LOQ: 0.5 ng/mL AEME in buffered oral fluid; LOQ in neat OF was 2 ng/mL (the dilution factor from collection device was 4) * Each sample was analysed in duplicate and values are presented as mean values.

Table 2. BZE concentration in oral fluid for each participant measured after the ingestion or swirling of a cup of coca tea.

	Concentration Group A (ng/mL) - Ingested coca tea								Concentration Group B (ng/mL) - Swirl coca tea															
	Collection time (min)								Collection time - Right side of mouth (min)								Collection time - Left side of mouth (min)							
Participant	0	10	20	30	60	120	180	240	0	10	20	30	60	120	180	240	0	10	20	30	60	120	180	240
P1	2	174	25	21	18	33	63	25	2	507	65	31	11	3	3	3	2	570	77	19	8	3	2	2
P2	2	61	62	13	30	16	10	21	2	584	77	27	9	3	3	2	2	646	107	35	10	3	3	2
P3	2	69	11	28	28	25	43	28	2	171	60	47	8	7	2	3	2	226	63	25	7	4	2	4
P4	1	34	30	15	31	0	4	9	2	329	408	15	10	3	2	2	1	219	287	24	6	4	3	2
P5	2	110	22	33	34	60	34	60	2	87	22	9	3	2	2	2	1	91	12	3	2	2	2	0
P6	1	13	18	16	24	25	18	18	2	1404	442	161	17	4	3	2	2	1175	219	87	25	3	3	1
P7	1	603	300	114	131	33	51	20	2	875	85	66	9	10	3	2	1	756	108	44	16	9	4	2
P8	1	58	43	22	27	23	25	23	2	290	33	9	4	2	2	2	1	366	34	7	2	2	2	1
P9	2	55	9	11	28	39	42	26	2	119	31	12	12	4	3	2	1	305	49	11	9	2	5	3
P10	2	363	108	25	34	29	11	28	2	913	27	12	3	2	2	2	1	1103	20	25	8	2	2	4
P11	1	31	19	19	15	17	11	21	2	214	44	9	10	2	3	2	1	78	18	4	4	2	2	2
P12	1	64	22	21	7	6	11	7	2	8	5	4	2	2	2	2	2	5	4	5	2	2	2	1
P13	1	9	6	4	7	4	2	2	2	416	129	9	4	2	2	2	1	557	23	11	11	2	2	2
P14	2	116	68	46	14	5	2	6	0	454	58	46	6	2	2	2	0	524	130	55	8	2	2	2
P15	1	16	8	7	4	2	2	2	2	152	97	43	9	2	3	3	2	294	56	24	7	2	3	3
Mean	2	118	50	26	29	21	22	20	2	435	106	33	8	3	2	2	2	461	80	25	8	3	3	2
Median	1	61	22	21	27	23	11	21	2	329	60	15	9	2	2	2	2	366	56	24	8	2	2	2
SD	0	161	75	26	30	17	20	14	0	380	134	40	4	2	1	0	0	352	81	23	6	2	1	1
Min	1	9	6	4	4	0	2	2	0	8	5	4	2	2	2	2	0	5	4	3	2	2	2	0
Max	2	603	300	114	131	60	63	60	2	1404	442	161	17	10	3	3	2	1175	287	87	25	9	5	4

Group A: Participants ingested one cup of coca tea (data collected using one collection device at each time point). Group B: Participants swirled a cup of coca tea (data collected using two collection devices at each time point). A: OF sample collected from the right side of the mouth. B: OF sample collected from the left side of the mouth. SD: Standard deviation. LOQ: 0.5 ng/mL BZE in buffered oral fluid; LOQ in neat OF was 2 ng/mL (the dilution factor from collection device was 4) * Each sample was analysed in duplicate and values are presented as mean values.

Table 3. Cocaine concentration in oral fluid for each participant measured after the ingestion or swirling of a cup of coca tea.

	Concentration Group A (ng/mL) - Ingested coca tea								Concentration Group B (ng/mL) - Swirl coca tea															
	Collection time (min)								Collection time - Right side of mouth (min)								Collection time - Left side of mouth (min)							
Participant	0	10	20	30	60	120	180	240	0	10	20	30	60	120	180	240	0	10	20	30	60	120	180	240
P1	2	1886	732	306	53	32	21	6	2	2703	592	166	35	6	6	6	2	3022	521	120	23	4	4	3
P2	5	1317	780	120	35	7	3	3	2	2437	733	206	40	3	3	2	2	2629	917	237	28	3	4	2
P3	9	1630	181	195	31	9	6	3	2	2683	1772	1320	179	48	5	3	2	2870	1656	844	112	22	2	3
P4	2	1242	912	397	145	2	2	3	1	2569	2073	146	68	10	3	3	1	2423	1822	206	39	9	9	3
P5	2	2137	1031	507	203	140	42	36	2	1554	324	122	24	4	4	3	2	1223	122	11	4	4	3	0
P6	1	737	236	56	31	10	6	3	2	4575	2837	1882	165	17	4	2	2	4643	2855	2172	205	10	4	1
P7	1	2993	2168	1082	184	5	4	2	2	3550	1301	999	166	45	7	3	1	3289	1900	874	276	37	9	2
P8	2	1115	449	89	26	4	4	3	2	2499	546	74	16	2	2	1	2	2458	566	39	6	1	2	1
P9	2	1215	158	105	41	8	4	3	1	2127	1292	442	198	12	3	2	1	2819	1510	509	133	6	5	2
P10	2	2359	627	74	30	8	3	15	1	3407	1195	150	15	2	2	3	1	3927	908	433	109	2	2	5
P11	2	207	80	43	22	10	4	4	1	2122	597	52	34	4	4	2	1	1628	191	19	23	4	3	2
P12	1	505	97	39	5	3	3	2	3	336	215	30	3	2	2	2	3	274	68	22	3	2	2	1
P13	1	58	14	4	3	2	1	1	1	3211	1933	156	45	5	2	2	1	2917	1421	666	109	2	4	2
P14	3	1176	641	346	103	8	2	4	17	2132	1259	723	66	2	4	2	17	2292	1488	803	73	2	4	2
P15	1	138	40	13	5	1	1	2	3	1730	1254	593	59	3	4	4	3	1958	1107	287	62	3	7	4
Mean	2	1248	543	225	61	17	7	6	3	2509	1195	471	74	11	4	3	3	2558	1137	483	80	7	4	2
Median	2	1215	449	105	31	8	4	3	2	2499	1254	166	45	4	4	2	2	2629	1107	287	62	4	4	2
SD	2	853	564	283	66	35	11	9	4	979	729	547	67	15	2	1	4	1055	779	563	79	10	2	1
Min	1	58	14	4	3	1	1	1	1	336	215	30	3	2	2	1	1	274	68	11	3	1	2	0
Max	9	2993	2168	1082	203	140	42	36	17	4575	2837	1882	198	48	7	6	17	4643	2855	2172	276	37	9	5

Group A: Participants ingested one cup of coca tea (data collected using one collection device at each time point). Group B: Participants swirled a cup of coca tea (data collected using two collection devices at each time point). A: OF sample collected from the right side of the mouth. B: OF sample collected from the left side of the mouth. SD: Standard deviation. LOQ: 0.5 ng/mL cocaine in buffered oral fluid; LOQ in neat OF was 2 ng/mL (the dilution factor from collection device was 4) * Each sample was analysed in duplicate and values are presented as mean values.

Table 4. EME concentration in oral fluid for each participant measured after the ingestion or swirling of a cup of coca tea.

	Concentration (ng/mL) - Ingested coca tea								Concentration (ng/mL) - Swirl coca tea															
	Collection time (min)								Collection time - Right side of mouth (min)								Collection time - Left side of mouth (min)							
Participant	0	10	20	30	60	120	180	240	0	10	20	30	60	120	180	240	0	10	20	30	60	120	180	240
P1	0	42	0	0	0	0	0	0	0	29213	12746	5877	1876	233	123	14	0	29275	12101	7003	1553	221	89	41
P2	0	56	8	0	5	0	0	0	0	17989	6418	2908	791	44	19	0	0	22222	8964	4697	726	60	23	2
P3	0	118	0	0	0	0	0	0	0	7152	6621	2724	324	240	58	56	0	7876	5424	2280	704	180	44	77
P4	0	161	41	33	0	0	0	0	0	12209	3181	1957	440	63	13	9	0	6758	2592	2160	243	52	7	0
P5	0	161	45	0	6	7	0	0	0	2946	966	472	164	28	32	8	0	2860	598	60	15	22	6	0
P6	0	0	0	0	0	0	0	0	1	31823	15551	7395	2804	191	43	8	1	26056	16561	10308	3239	139	63	0
P7	0	382	193	88	0	0	0	0	0	14630	4446	3789	586	234	68	33	0	13032	6421	3682	569	184	75	31
P8	0	150	10	9	0	0	0	0	0	15135	6256	3774	721	158	43	26	0	13913	3599	3563	407	191	49	34
P9	0	65	12	0	16	0	0	0	0	4476	468	769	0	68	0	0	0	8970	76	52	218	0	29	0
P10	0	8911	2495	859	250	48	0	0	0	10937	1027	3657	136	12	91	26	0	20025	406	3660	18	113	44	24
P11	0	1354	779	383	92	11	0	0	0	2161	1400	1333	428	0	21	0	0	337	1463	957	20	5	0	0
P12	0	4101	1610	433	87	16	4	0	0	6256	99	111	638	58	0	0	0	196	60	3763	460	0	0	0
P13	0	1802	387	206	17	0	0	0	0	12374	9985	5480	188	123	0	0	0	27359	317	672	1544	0	11	53
P14	0	6560	4181	3194	450	7	0	0	2	4287	2931	940	141	0	1	0	2	6070	3344	1506	218	3	3	0
P15	0	1976	2352	637	72	76	58	11	0	4704	2183	824	227	12	1	1	0	4085	1606	697	199	11	7	0
Mean	0	1723	807	389	66	11	4	1	0	11753	4952	2800	631	98	34	12	0	12602	4235	3004	676	79	30	18
Median	0	161	45	33	6	0	0	0	0	10937	3181	2724	428	63	21	8	0	8970	2592	2280	407	52	23	0
SD	0	2725	1277	822	126	22	15	3	1	9051	4689	2195	753	91	37	17	1	10037	4919	2801	860	83	29	25
Min	0	0	0	0	0	0	0	0	0	2161	99	111	0	0	0	0	0	196	60	52	15	0	0	0
Max	0	8911	4181	3194	450	76	58	11	2	31823	15551	7395	2804	240	123	56	2	29275	16561	10308	3239	221	89	77

Group A: Participants ingested one cup of coca tea (data collected using one collection device at each time point). Group B: Participants swirled a cup of coca tea (data collected using two collection devices at each time point). A: OF sample collected from the right side of the mouth. B: OF sample collected from the left side of the mouth. SD: Standard deviation. LOQ: 1 ng/mL EME in buffered oral fluid; LOQ in neat OF was 4 ng/mL (the dilution factor from collection device was 4) * Each sample was analysed in duplicate and values are presented as mean values.

APPENDIX G - Absorption of cocaine in tongue tissue

Table 1. Amount of cocaine absorbed in porcine tongue tissue following exposure to 200 mg crack cocaine.

Experiment	Site of tongue	COC concentration (ng/mL)			SD	%RDS
		Mean	Min	Max		
TONGUE 1	Top (*n* = 7)	53.1	26.0	94.6	25.4	47.7
	Middle (*n* = 8)	107.2	58.2	231.4	55.7	51.9
	Low (*n* = 5)	140.3	71.3	206.8	59.7	42.5
TONGUE 2	Top (*n* = 6)	7.3	6.1	9.6	1.3	17.2
	Middle (*n* = 6)	7.2	3.4	9.0	2.6	36.6
	Low (*n* = 5)	12.7	3.5	26.0	9.5	75.2
TONGUE 3	Top (*n* = 6)	12.6	8.2	24.1	5.9	47.2
	Middle (*n* = 9)	15.2	7.4	21.6	5.3	34.8
	Low (*n* = 5)	10.5	7.9	15.5	3.0	29.0

COC: Cocaine; SD: standard deviation; %RSD: Percentage standard deviation, *n* = number of subsamples analysed.