TALLINN UNIVERSITY OF TECHNOLOGY
School of Information Technologies
Department of Health Care Technology

Chelsa Ann Brilla A156294

RAPID DETERMINATION OF AMPHETAMINES IN SALIVA BY PORTABLE CAPILLARY ELECTROPHORESIS

Master's thesis

Supervisor: Jekaterina Mazina-Šinkar
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Researcher

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Chelsa Ann Brilla A156294

AMFETAMIINIDE KIIRE MÄÄRAMINE SÜLJES PORTATIIVSE KAPILLAARELEKTROFOREESI SEADMEGA

Magistritöö

Juhendaja: Jekaterina Mazina-Šinkar
Doktorikraad
Teadur

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Author’s declaration of originality

I hereby certify that I am the sole author of this thesis. All the used materials, references to the literature and the work of others have been referred to. This thesis has not been presented for examination anywhere else.

Author: Chelsa Ann Brilla

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Abstract

The aim of this thesis was to develop and validate a quick and robust method to test and quantitate the amount of amphetamine (AMP) and methamphetamine (METH) in saliva using portable capillary electrophoresis (CE) with a fluorescence detector. Current methods of testing for illegal drugs are usually lacking in one of two common areas, matrix concerns or instrument and processing limitations for outside of the lab testing. The method developed in this thesis addresses those challenges.

A starting point for the method was found using currently published research and methods used and then tested and optimized for the specific conditions required by the hypothesis and adjusted for the needs of the portable CE instrument. This method was developed from the beginning to be compatible with simultaneous testing of cocaine, cocaethylene and some ecstasy analogues. The European Medicines Agency (EMA) *Guidelines on Bioanalytical Method Validation* was chosen as a guide for validating this method. Components of the validation able to be fully completed included selectivity, carry over, and lower limit of quantification. Other sections required modification due to lack of AMP and METH to work with. These included calibration, accuracy, precision, matrix effect and stability. Dilution integrity could not be modified enough without losing the intent of the test and was therefore not tested to the standard.

The validity of the optimized method was confirmed using saliva samples of suspected drug users collected by the Pärnu Police Department during the Weekend Music Festival which took place on the 4-5th of August 2017, in Pärnu, Estonia. In total, 37 samples were collected and analyzed for illegal drug abuse. This study presents the results of five positive samples. The results of the entire project are going to be submitted for publication in a peer reviewed journal by the illegal drug team at Tallinn University of Technology in 2018.

This thesis is written in English and is 32 pages long, including 6 chapters, 15 figures and 4 tables.

Annotatsioon

Käesoleva töö eesmärk oli välja töötada ja valideerida kiire ning robustne meetod kvantitatiivseks amfetamiini (AMP) ja metaamfetamiini (METH) määramiseks süljeproovidest kasutades kapillaarelektroforeesi (CE) fluorestsentsdetektoriga. Tallinna Tehnikaülikooli narkootiliste ainete uurimisrühm seadis hüpoteesi, et on võimalik kiirelt ning selektiivselt kvatntifitseerida amfetamiine süljeproovides 10 minutiga, kasutades selleks uudset kapillaarleketroforeesi seadet, kus proovi kogumine ning ettevalmistus oleks suhteliselt lihtne. Sekundaarseteks eesmärkides olid: amfetamiinide määramine samaaegselt teiste narkootiliste ühenditega, hinnata erinevaid proovi kogumise katsuteid ning määrata selektiivsus tarvitatavate ravimite suhtes.

Kasutusel olevate narkootiliste ainete analüüside puhul on kõigil puudujääke ühes või kahes peamises etapis. Mitmed analüüsimeetodid kasutavad uriini või verd, mis tekitavad tõsiseid küsimusi invasiivsuse, privaatsuse ning proovi võltsimise osas. Osa väga selektiivseid meetodied, mis kasutavad sülge proovimaatriksina, vajavad instrumente või ettevalmistusetappe, mida on väga keerukas väljaspool laborit rakendada. Käesolevas töös välja töötatud meetodil ei esine antud puudujääke.

Alustuseks töötati läbi seni avaldatud teadusartikleid ja kasutusel olevaid meetodeid, mida omakorda prooviti ning optimiseeriti spetsiifiliselt töös esitatud hüpoteeside tingimuste täitmiseks ja seejärel kohandati portatiivsele CE instrumendile. Käesolev meetod töötati algusest peale välja samaaegseks kokaiini, kokaetüleeni ja mitme *ecstasy* analoogi analüüsiks. Antud meetodi valideerimiseks valiti Euroopa Meditsiiniagentuuri (EMA) Bionanalüütilise Meetodi Valideerimise Eeskiri (inglise k. *Guidelines on Bioanalytical Method Validation*). Täielikult viidi läbi valideerimine selektiivsusele, ülekanduvusele ning alam-määramispiirile. Teised parameetrid viidi läbi osaliselt või muudetud kujul vähese AMP ja METH standardi hulga tõttu. Nendeks olid kalibratsioon, kordustäpsus, täpsus, maatriksi efekt ning stabiilsus. Hindamaks lahjendusefekti, tuli parameetri määramist standardi koguse hulga tõttu modifitseerida niivõrd, et kaotas see oma sisu, seega antud parameetrit ei hinnatud.

Optimeeritud meetodi valideerimise kinnitamiseks rakendati meetodit Pärnu Politseijaoksonna poolt kogutud narkootikumide tarvitamises kahtlustatute süljeproovidele, mis koguti Weekend Music Festivali ajal 4.-5. august Pärnus, Eestis. Kokku saadi ning analüüsiti 37 proovi tuvastamaks narkootiliste ainete tarvitamist, käesolev töö esitleb neist viit positiivset proovi. Kogu projekti tulemused avaldatakse välisekspertide poolt ülevaadatud rahvusvahelises teadusajakirjas Tallinna Tehnikaülikooli narkootikumide uurimise rühma poolt aastal 2018.

Lõputöö on kirjutatud inglise keeles ning sisaldab teksti 32 leheküljel, 6 peatükki, 15 joonist, 4 tabelit.

List of abbreviations and terms

ACN	Acetonitrile
ACP	Allocryptopine
AMP	Amphetamine
BENZ	Benzylamine
BGE	Background Electrolyte
C4D	Capacitively coupled contactless conductivity detector
CE	Capillary Electrophoresis
DOA	Drug of Abuse
DRUID	Driving Under the Influence of Drugs, Alcohol and Medicines Project 2008-2012
DUID	Driving under the Influence of Drugs
EMA	European Medicines Agency
EMCDDA	The European Monitoring Centre for Drugs and Drug Addiction
EU	European Union
FDA	United States Food and Drug Administration
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
IDL	Instrument Detection Limit
IQL	Instrument Quantification Limit
IS	Internal Standard
IUPAC	The International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MDA	3,4-Methylenedioxyamphetamine
MDEA	3,4-Methylenedioxy-N-ethylamphetamine
MDMA	Methylenedioxymethamphetamine
METH	Methamphetamine
MS	Mass Spectroscopy

TRIS	Tris(hydroxymethyl)aminomethane
TTU	Tallinn University of Technology
USFDA	United States of America Food and Drug Administration
UV	Ultraviolet

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1 Introduction

The illegal drug testing team at Tallinn Technical University is working on developing new and novel testing methods for commonly abused drugs as well as new designer drugs. The focus is to develop a portable illegal drug of abuse (DOA) analyzer for illegal drugs using oral fluid and capillary electrophoresis (CE) coupled to a fluorescence detector (FD). Several studies have determined that some of the most commonly abused drugs in Europe in the past few years are amphetamine, methamphetamine, cocaine and ecstasy analogues [16] [12] [14]. Despite perceptions to the contrary, alcohol is regularly ingested with these drugs as well, also affecting impairment [16]. The drugs for this research: methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), cocaine, cocaethylene and amphetamine, and methamphetamine, were specifically chosen due to their high level of abuse and regular appearance in random drug screens of drivers. Amphetamine (AMP) and methamphetamine (METH) are some of the most commonly abused drugs of choice around the world when available [3] [6] [12] [16]. Ecstasy analogues (MDA, MDEA) have become so popular that MDA and MDEA were added to the US's Federal Workplace Drug Testing Guidelines in 2016 [4].

One of the objectives of the team is to develop methods for testing common illegal drugs on the portable DOA analyzer within a quick timeframe. This would allow law enforcement to be able to prove illegal drugged driving or provide enough evidence quickly to warrant a blood exam. It could also be used in drug rehabilitation centers for confirmation that patients are following proscribed treatments as well as in athletic competitions to quickly confirm the absence of performance-enhancing amphetamines and ephedrines [15]. There are countless other applications for which users of this machine might want quick, on-the-spot results of drug analysis using saliva as a non-invasive diagnostic tool.

As a result of this need, the hypothesis of the thesis is that it is possible to develop a quick and robust method to test and quantitate the amount of amphetamine (AMP) and methamphetamine (METH) within 10 minutes, from saliva samples using portable capillary electrophoresis (CE) with a fluorescence detector and simple sample processing.

The method characteristics will be compared to the validation process for the European Medicine Agency's (EMA) guidelines on bioanalytical method validation (EMA/275542/2014). Although the focus will be on amphetamines, the method will be developed to test amphetamines simultaneously with other commonly abused illegal drugs (specifically cocaine, cocaethylene, MDMA, MDA, MDEA and others). Secondary objectives include studying the selectivity of the method against different prescription medications and comparing the efficiency of two different Salivette® collection tube swabs with a cotton swab and with a synthetic swab, choosing the best one for simple and efficient sample collection, extraction, and pre-concentration.

2 Literature review

This review shares the current knowledge of amphetamine and methamphetamine, testing for these drugs as well as details on testing.

2.1 Illegal drug trends

Testing for illegal substance use has been in effect world-wide for decades. Numerous groups and agencies have different purposes for testing for the use and abuse of drugs and alcohol. The most common areas of importance are to law enforcement, employers, physical competitions and rehabilitation clinics. As the categorization, development and differing trends in abuse have changed over the course of time, so too, has the need for different types of testing methods and equipment to positively identify and quantitate these substances.

Employers and government agencies want to show no immediate drugs of abuse in the system that could put employees or customers in danger. The government of the United States first published it's Mandatory Guidelines for Federal Workplace Drug Testing Programs in 1988 [5]. Rehabilitation clinics need quick and effective testing to confirm that patients are following all courses and not abusing prescriptions. Athletic competitions test competitors to ensure no cheating is taking place. Law enforcement and hospitals not only need exacting quantification of type and amount of substance for treatment and legal admission in court cases, but also have a need to determine quickly if a person suspected of driving under the influence of drugs is actually impaired and a danger to others.

As awareness and consequences for drunk driving are increasing around the world, a newer danger is arising from drugged driving [9]. In October, 2006, the DRUID project (Driving Under the Influence of Drugs, Alcohol and Medicines) was created to provide scientific data to policy makers to help understand the actual situation in the European Union. Their data was published in 2011 and 2012 [7]. Within the EU, the DRUID project calculated that on average, "3.48 % of drivers in the European Union drive with alcohol (> 0.1 g/l) in their blood, 1.9 % with illicit drugs, 1.4 % with (a limited list) of medicinal drugs, 0.37 % with a combination of alcohol and drugs, and 0.39 % with different drug classes" [4]. Although the focus of many accident prevention programs is still on alcohol,

there is a persistently growing need for more consistent, economical, quick and easy-to-use drugged driving testing methods [9].

2.2 Pharmacokinetics of amphetamines in blood and urine versus oral fluid

Amphetamine (AMP) is a central nervous system stimulant with a chemical formula of $C_9H_{13}N$ (Figure 1A). The IUPAC identification is *N*,α-methylbenzeneethanamine. It is a member of the phenethylamine family along with methamphetamine. Methamphetamine (METH) has a chemical formula of $C_{10}H_{15}N$ and an IUPAC identification of *N*,α-dimethylbenzeneethanamine. (Figure 1B). During synthesis, both generally produce racemic mixtures of the R- and S- enantiomers, although AMP does have a less commonly used method of synthesis that can give a stereoselective end product. It is possible to determine the synthesis pathway through the analysis of the impurity profile.

Both are typically found as a white powder, although the crystalline hydrochloride form of METH is also regularly made and is referred to as ICE. AMP and METH may be ingested, snorted and injected while METH can also be smoked. Both AMP and METH have been used occasionally as legal and prescribed medications for narcolepsy and attention deficit hyperactivity disorder (ADHD). Street names for AMP include speed, base, and whiz; while METH goes by the street names speed, crank, meth, crystal meth, pervitin and yaba. The most common adulterants (cutting agents) for both are caffeine, glucose, ephedrine and ketamine. METH has also been found to be an adulterant in ecstasy (MDMA) [1][2].

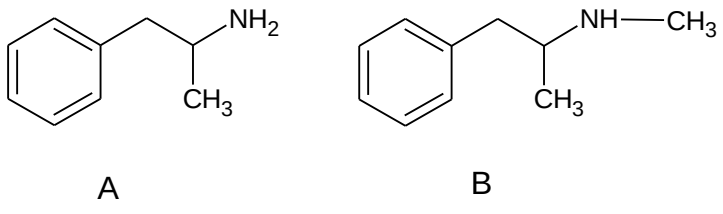

Figure 1. A – Amphetamine [1] B – Methamphetamine [2]

AMP and METH both work as psychostimulants by increasing the release of dopamine from nerve terminals, inhibiting the metabolism of dopamine and increasing the release of noradrenaline and serotonin. This typically results in euphoria, mood elevation, an

increase in energy, concentration and focus, and improvement in physical performance. [25]

When testing blood and oral fluid of subjects given AMP and METH in controlled environments, it can be seen that the concentration peaks at approximately the same time and follows similar patterns of concentration levels. [41] See Figure 2 for comparisons of subjects dosed with 10 and 20 mg sustained release methamphetamine. The main difference is that oral fluid tests at higher concentrations than blood. (Up to 325 µg/L for METH and up to 22.5 µg/L for AMP in oral fluid and up to 40 µg/L for METH and up to 12.5 µg/L for AMP for blood). Studies such as the Gjerde and Engblom studies continue to show a high correlation between AMP and METH in blood and oral fluid. [39], [42] Figure 3 A and B demonstrates this correlation. Figure 3 A compares the concentration of AMP in oral fluid to the concentration of AMP in whole blood of people confirmed as driving under the influence of drugs (DUID) during the Roadside Assessment Testing Project (ROSITA-2). [42] Figure 3 B shows the correlation found between the concentration of AMP in blood and oral fluid in drivers suspected of DUID as well as patients admitted to acute psychiatric treatment (both in Norway). [39] These studies also confirm that the concentration levels of AMP and METH are higher in oral fluid (up to 125 mg/L) then in blood (up to 3 mg/L). However, there is no proven direct relationship whereby one can state the concentration in the blood based solely on the amount of AMP and METH in oral fluid. What the studies do consistently show is that a separate calibration and cut-off allowance can be determined for the method involving oral fluid which can be then quantified as DUID. This is one of the reasons the team at TTU is studying oral fluid testing on their portable CE.

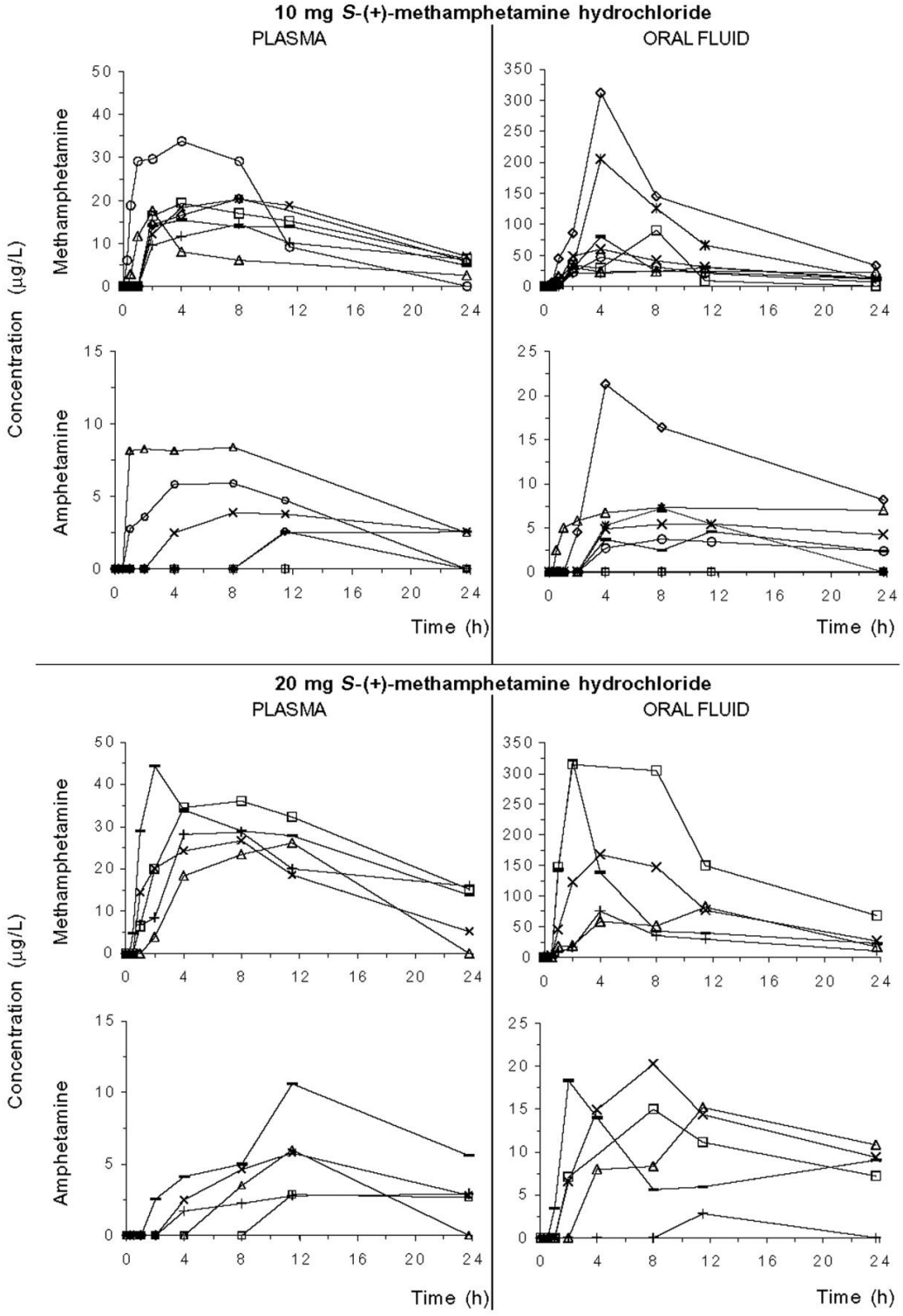

Figure 2. Pharmacokinetics of AMP and METH from a controlled dosage study [41]

"Drug concentration profiles in plasma (left) and oral fluid (right) after administration of 10- and 20-mg sustained release S-(+)-methamphetamine hydrochloride. All eight volunteers received the first low dose, but no plasma specimens were available for Z (∗). Volunteers S (□), W (□), Y (×), AA (+), and BB (–; n = 5) received both the 10- and 20-mg doses. ⋄, volunteer V; ○, volunteer X. Oral fluid samples were obtained after stimulation of expectoration with citric acid candy." [41]

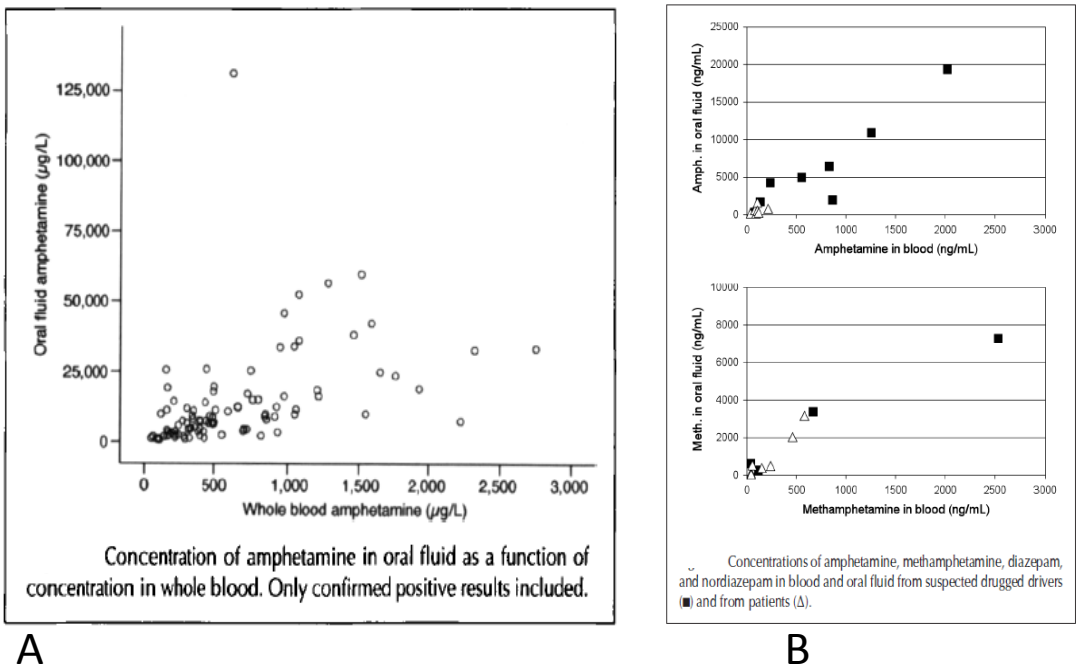

Figure 3. A. Relationship of AMP oral fluid and blood concentrations of confirmed DUID in Norway [42] B. Relationship of oral fluid and blood concentration of AMP and METH in suspected DUID and acute psychiatric patients from the ROSITA-2 project [39]

2.3 Sample matrices as a tool for diagnostics

For years, the standard sample matrices for drug testing were blood and urine. These provided the original drug test developers high amounts of drugs in the samples, semi-consistent matrices and relatively long stability of the drug before breaking down or being excreted. However, blood tests are considered highly invasive, and the laws around rights and refusals for blood tests are extremely complex and highly location dependent. In many places, reasonable suspicion is not enough for law enforcement to demand a blood test, a warrant must be issued, increasing the time from accusation/suspicion until testing and, therefore, decreasing the amount of the drug actually in the person's system.

Voluntary submission of blood tests, especially within the United States, is extremely low and can lead to underrepresentation of actual drugged driving numbers [6]. Drawing blood is also the riskiest of the currently accepted procedures. Any time a person is punctured for a blood draw, there is always a medical risk of infection or complication. The risks to those processing the blood sample are also much higher than other sample matrices due to the possibility of blood-borne illness infection. Despite risk management

plans, sharps injuries and blood-borne infection of healthcare workers continue to be a serious concern. In one study of healthcare workers in the UK over a 10-year period, 2947 sharps injuries were reported. Of those sharps injuries, 49% were associated with a hepatitis C infected patient, 23% were associated with a HIV infected patient and 15% were with patients whom the infection status was unknown at the time [36]. The World Health Organization even has an occupational health program for the approximately 2 million healthcare workers worldwide, who experience needle-stick injuries on the job [37].

As such, urine testing has been the fall back for many law enforcement officers, athletic competitions and employee screening tests. But there is still a privacy concern over the collection methods. In most places, urine collection must be done in private or with an observer of the same gender. It is also easily adulterated, not only affecting the drug amounts, but often invalidating the entire test.

The internet is full of advice, products and methods for people to try to fool a drug screen or invalidate the test, giving the drug more time to leave their system and ultimately costing more time and money [19]. This is why in recent years, there has been a push for a change in test matrices and alternative, quick, cheap and reliable test methods. Other matrices being heavily investigated include oral fluid, sweat, dried blood spots, hair, ocular fluid (for the deceased) and more. Currently, any method that utilizes dried blood spots involves a more rigorous validation through the USFDA as it is not widely accepted yet [18].

Scientists in the illegal drug testing arena have gravitated towards oral fluid and hair due to their ease of collection and difficulty in altering. One of the main disadvantages to hair though, is the need for considerable pre-treatment and concentration prior to testing [11]. Oral fluid can be collected by those with limited training, it poses considerably less danger to those collecting (unlike with blood), no expensive special equipment is needed and it is cost effective for mass screening [13] [22]. There are, however, some drawbacks to using oral fluid. It can be difficult to collect samples in large volumes (due to xerostomia), the high viscosity of oral fluid can cause issues in pipetting and some drugs simply have too short of a plasma half-life to make testing reasonably functional. Despite the disadvantages, in many places the testing of oral fluid is now widely accepted and part of

a standardized methodology for illegal drug testing [4] [6]. This solves many of the concerns over the invasiveness of blood samples and the privacy and purity concerns over urine samples.

Oral fluid is made up of saliva from the various oral glands (major and minor) as well as other constituents in the mouth such as food debris, bacteria, nasal secretions and others. Whole saliva contains various inorganic molecules like water, Na^+, K^+, $Mg^{2+,}$ Ca^{2+}, Cl^-; multiple organic compounds such as amino acids, lipids, and glucose; as well as hormones [13]. In general, the oral cavity has a very rich blood supply which allows the drugs to pass into saliva through passive diffusion. Because lipid-soluble molecules diffuse through the cell membranes easier, it is typical for the whole drug (not protein bound, and unmetabolized) to diffuse. It works best for drugs that are not ionizable within the normal pH range of saliva as once the drug is ionized or bound, it is not permeable to the cell membrane [4]. Therefore, it is often the parent drug that is usually present in the oral fluid.

This drug amount is correlated to the amount of free drug in blood plasma although the correlation is specific to each drug and is also strongly affected by the acidity of the drug [4], [10]. According to the Mercolini and Protti study though, this is the "ideal concentration" because it mirrors the concentration of the drug at the biologically active sites in blood plasma [10]. And because the transfer of the drug from blood to oral fluid is quick, time since application does not pose a problem for testing, particularly for amphetamines, as "ion-trapping" occurs due to the oral fluid's higher acidity. The opposite concern, that of concentrations being higher due to oral ingestion, smoking or other related application method is generally allayed by the fact that the oral cavity has a well-developed self-cleansing mechanism [4], [10], [13].

Another advantage that oral fluid testing has in comparison to urine testing is that urine based drug testing generally looks for metabolites of the drug. Although quantifiable for positive/negative recent drug use, urine testing cannot be used to determine current level of impairment [4], [22]. Oral fluid testing also has the advantage over urine testing for new drugs on the market. Urine based metabolites may not be known yet but since oral fluid tests typically look for the parent drug, new methods can be studied as soon as the need arises [15].

2.4 Paired Samples

Although not abundant, there have been studies done comparing positivity rates of oral fluid to blood or urine samples. The gold standard method of these studies is to utilize "paired" specimen collection instead of the comparison of overall positivity rates. Paired collection is defined as collecting two samples (oral fluid, blood, urine, hair) from the same subject as near simultaneously as possible [4]. This allows for the method under investigation to be compared to a method that is currently in use, usually oral fluid to blood or oral fluid to urine. In a 2012 study published in the *Journal of Analytical Toxicology* by Heltsley *et al.* that compared oral fluid to urine samples in pain management patients, it was found that there was an 85% agreement in the test results of over 1500 paired tests [27].

In a different type of study by Wish and Yacoubian Jr from the journal *Federal Probation*, urine tested illegal drug rates were compared to oral fluid tested drug rates of Baltimore city arrestees. In that study, high sensitivity and specificity (above 90%) were determined for most of the illegal drugs tested where there were enough positives to calculate reasonable rates [28]. As previously stated, there is no direct calculation that can be done to "convert" the cut-off limits of one type of test to another. This is shown in the 2010 Gjerde study where paired oral fluid and blood samples were compared to each other. The study concluded that the variations did not allow reliable estimates of drug concentrations in blood based on oral fluid. [39] The 2007 Engblom tested 153 paired samples that used different methods and cut-offs and found only seven samples that tested positive for AMP in oral fluid but not in whole blood. (It should be noted that there were 39 samples unable to be compared due to insufficient amounts of oral fluid.) However, all seven of the AMP false-positive samples did test positive for other illegal drugs in whole blood. [42]

2.5 Current methods of testing

In the majority of situations however, the oral fluid samples are collected and then sent away for testing in an approved analytical lab. Depending upon an infinite number of variables, it could be days, weeks or even months before a result is known and reported to law enforcement, employers or other requestors. Currently, the most common methodologies for testing oral fluid are done with gas chromatography (GC), liquid

chromatography (LC), mass spectroscopy (MS) and various combinations of these three such as GC-MS or LC-MS. These instruments, which are capable of determining the type of drug, quantifying the amount and possibly creating a chemical profile, are very expensive to purchase, run, maintain and require specially trained personnel. This is why there is a considerable market need for cheap, quick and rugged drug testing equipment, similar to the Breathalyzer method for alcohol abuse. One study noted that this could be particularly helpful in underdeveloped countries with limited financial resources, extremely long response times from overburdened laboratories and where low levels of expertise exist [14].

Currently, there are multiple quick assay tests that need little training, in which police officers on-scene can use to test suspected substances (not the person). These one-time use immunoassays and color change reactions have so far dominated the market (as opposed to portable Raman spectroscopy or Fourier Transform Infrared Spectroscopy – FTIR), but newer more objective methods are currently being developed and are highly sought after [7]. These methods however, still don't indicate if the driver is currently compromised and driving under the influence of drugs. A product called "DrugWipe" is currently on the market as a substance, saliva and sweat test for AMP and METH [26]. It claims that using the saliva and sweat option, it can prove that the driver is under the influence, although it does not quantitate the drug. The website lists results all above 95% for sensitivity, specificity and accuracy from their own laboratory quality testing, however, outside studies are mixed on confirmation of such positives. As part of the DRUID project, "Drugwipe" was tested in Finland in 2010 and gave an 87% sensitivity, 95% specificity and 93% accuracy result for AMP [9]. However, a 2016 study of club-goers in Rome found these rates to be closer to 80% for all 3 capabilities. Of the 83 DrugWipe tests compared to matching oral fluid samples tested using a standard GC-MS method, 14 were false negatives or false positives for AMP. Only cannabis gave lower match-ups [8], [26]. Unfortunately, these positive identification tests are usually acceptable as a screening method to grant law enforcement justifiable cause for further testing to determine, if DUID. So, although some quick and portable methods for detecting illegal drugs in oral fluid have been developed, currently approved methods for impairment identification, determination and quantification still utilize instruments that are not cheap or portable, need improvement on sensitivity, specificity and accuracy or require considerable expert training to use and interpret the results.

2.5.1 Capillary Electrophoresis

Capillary electrophoresis is one of the most robust, adjustable and up-and-coming instruments in analytical chemistry. At the basic level, a capillary electrophoresis instrument contains two reservoirs of a background electrolyte in contact with an anode and a cathode, connected via a capillary with a viewing window in which a detector is positioned (Figure 4). The system is then charged with an electric current and samples separate based on the electroosmotic flow of the sample's constituents. This simplicity makes it ideal for development of separation methods.

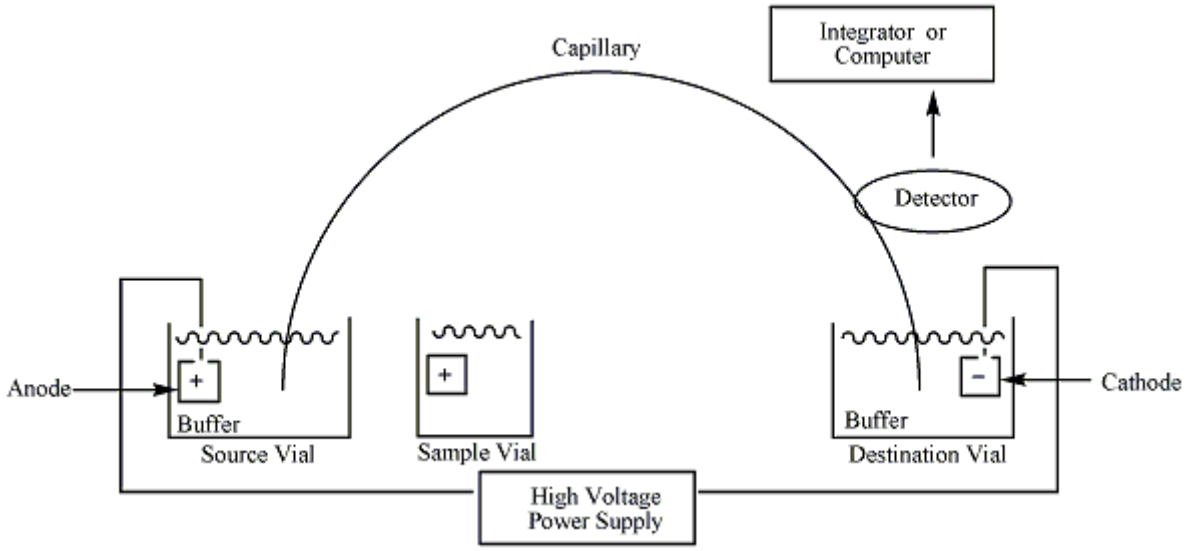

Figure 4. Diagram of a basic capillary electrophoresis system [21]

There has been interest in using CE for illegal drug testing since 1991 when Weinberger and Lurie compared a quick CE method to that of a well-developed and optimized HPLC system with highly successful results [23]. In the past, CE has been successful in determining the presence of MDMA in tablets as well as in other drugs and mixtures [11],[14]. Considering the extremely low amount of sample needed and the economical use of reagents, there is considerable benefit to developing CE methods for drug testing as opposed to using other instruments and systems. The problem with CE has traditionally been that using the most common detectors, ultra-violet (UV) detectors or capacitively coupled contactless conductivity detectors (C4D), the test simply does not get a high enough response due to low sample injection volume and the short pathlength of the detector [20]. However, many of the drugs of abuse sought for detection can be used with a fluorescence detector without much derivatizing and response issues become muted when using fluorescence [11].

2.5.2 Detectors

Over time, several detectors have become commonplace in the realm of bioanalytical and chromatographic testing. For instruments such as the gas chromatograph or the mass spectrometer, the detectors are usually destructive of the sample and quite different than those used in capillary electrophoresis and liquid chromatography. The most common detectors for capillary electrophoresis, especially for saliva testing are ultra-violet-visible (UV-VIS) or diode array (DAD), and capacitively coupled contactless conductivity (CE-C4D) [20]. Other non-destructive chromatographic detectors include refractive index, electron capture and fluorescence. Each detector utilizes a different chemical property and has specific advantages and disadvantages to it.

Many of the drug testing methods currently utilize absorbance detectors. This means that it exposes the sample to energy and measures the amount of energy absorbed. That absorption amount is then correlated to a concentration in a linear manner. Once a calibration curve is developed and the linear formula known, the concentration of an unknown sample can then be back-calculated using the amount of energy absorbed. Fluorescence detectors on the other hand are emissions detectors. They expose the sample to energy, which it absorbs and then measures the emission of that energy when it is released by the substance. Although most compounds will absorb at various wavelengths of energy, a vast majority of them do not fluoresce because the molecules have the ability to release the energy in non-radiative transitions at a greater rate than fluorescence [33]. Figure 5 demonstrates these three actions on the electron level when a compound is exposed to energy in the form of electromagnetic radiation [32].

Compounds with rigid ring structures fluoresce the best although most aromatics will fluoresce to some degree. Fluorescence can be measured by quantum yield, which is the fraction of excited molecules returning to the ground state by fluorescence. It ranges from 1, where all of the molecules return via fluorescence, to 0, where none do (zero fluorescence) [34]. There are some strong advantages to using fluorescence over absorption in conjunction with capillary electrophoresis, despite the relative rarity of inherent fluorescence. Molecular fluorescence spectroscopy is more sensitive, often 1 - 3 orders of magnitude greater than absorption spectroscopy. It also tends to have a much

larger linear concentration range, which is important to this project due to the extreme variations of drug amounts in an individual's system [33].

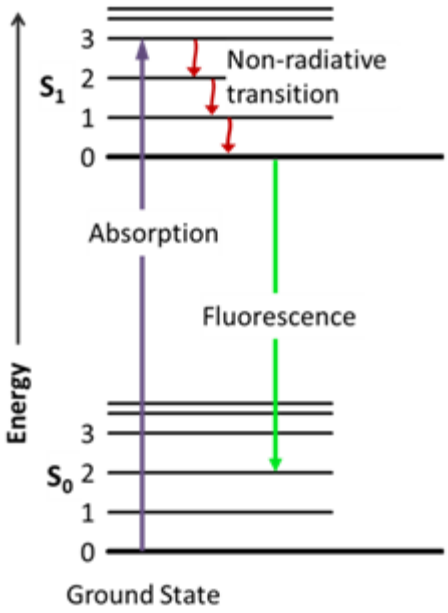

Figure 5. Energy diagram showing the difference between absorption and fluorescence on a molecular level. S_0 and S_1 are molecular orbitals, the arrows represent electrons moving between orbitals. [37]

2.6 CURRENT LEGISLATIONS

Over the past two decades, countries around the world have been updating their laws and regulations to better limit and prohibit DUID. Although laws against drinking and driving as well as legal limits have been established for a long time, the need has arisen for specifying what exactly constitutes DUID. Several projects such as the DRUID project and the ROSITA-2 project were established to research this problem [5], [30]. The additional complication to establishing legal limits is how the testing is done. As explained previously, cut-off limits for blood do not directly correspond to cut-off limits in urine or saliva. Different legal codes also mandate different methods of specimen collection, privacy protections and other challenges.

In Finland, the ministry of the interior has established that on site oral fluid tests for drugs are equivalent to on site Breathalyzer tests for alcohol [30]. Several countries have gone so far as to establish exacting legal maximums for AMP and METH. Germany has set the limit at 25 ng/ml of AMP in blood, and is "zero-tolerance" in intention [30]. Norway has set the AMP limit to 41 ng/ml and the METH limit to 45 ng/ml in blood [3]. Norway is

an interesting case as there have been several studies and suggestions to correlate the legal blood limits to oral fluid limits. In studies performed by the Norwegian Institute of Public Health, differing calculations and comparison methods have found various numbers. One method equated the blood levels to a limit of 190 ng/ml AMP and 270 ng/ml METH in oral fluid. However, the same study notes that using a different calculation method, the blood limits correlated to 300 ng/ml AMP and 200 ng/ml METH [6]. The U.K. has gone so far as to categorize the drugs, recognizing that AMP can have a positive effect on driving impairments like fatigue and slow reaction times, up to a point. So, in the U.K. there is a "zero-tolerance" limit on METH in blood samples of 10 ug/L, but a maximum "separate approach" limit of 250 ug/L of AMP. [31] Many countries, like Australia, have decided to eliminate the need for more research and simply state that any presence of illegal drugs in the system, blood or oral fluid is illegal [38]. All of these countries also have differences in the legal code about whether drivers are mandated to provide oral fluid samples upon request by police, if they can be charged using oral fluid tests results and if a secondary confirmation is needed from another method. The differences in laws, calculations and methods around the world demonstrate that while countries are in the process of updating their laws and procedures, more research on drugged driving limits, testing methods and oral fluid to blood correlations is needed.

2.7 Bioanalytical method validation

2.7.1 European Medicines Agency and the USA Food and Drug Administration

The benchmark for any analytical research completed that strives to actually be useful in the western world is to be certified under the bioanalytical method validation program from the European Medicines Agency or the USA Food and Drug Administration. Since this program works with drug testing, in order for it to be put to use in a real-world scenario, it would need to be validated under one of these two agencies. According to the European Medicines Agency, "The main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix, such as blood, serum, plasma, urine, or saliva." [17].

For the sake of thoroughness, two documents from the FDA are listed in the discussion of method validation. At the time of research, the most recent finalized guidance was

published in 2001. On the official website is a draft guidance published in 2015. The FDA was contacted via a *"contact us"* link to confirm that the published 2015 draft guidance is still in process. The FDA responded, affirming that the 2001 guidance is still official and that the 2015 guidance is still categorized as draft and the timeline for final approval is unknown [24] (See appendix 1 for full email transcript). There are not many differences between the 2001 version and 2015 draft version. The 2015 draft version includes more in-depth definitions, several mathematical formulas absent the previous version and a section on New Technologies that specifically addresses new and novel research methods. Overall, the EMA and USFDA documents describe similar procedures and parts of a method validation. The basic sections for method validation are shown in Table 1 below. Although it appears that the EMA guidelines have more requirements, most of the additional items on the list are actually part of a differently named section in the USFDA guidelines.

Table 1 Comparison of steps in EU vs. US bioanalytical method validation [17] [29] [18]

European Medicines Agency	**USA Food and Drug Administration 2001 Final**	**USA Food and Drug Administration 2015 Draft**
Selectivity	Selectivity	Selectivity
Carry Over	Accuracy, Precision & Recovery	Accuracy, Precision & Recovery
Lower Limit of Quantification	Calibration / Standard Curve	Calibration Curve
Calibration Curve	Stability	Sensitivity
Accuracy		Reproducibility
Precision		Stability
Dilution Integrity		
Matrix Effect		
Stability		

2.7.2 Design of Experiment

When developing a design of experiment for this thesis, the EMA guidelines on method validation were used to determine the primary steps, control factors and passing guidelines for this particular method validation. Each section will briefly be covered; however, appendix 2 contains the details of the spreadsheet that was designed to assist with planning all of the testing needed for validation.

Selectivity testing is used to test that the method can differentiate the amphetamine, methamphetamine and internal standards from any existing compounds in the chosen matrix. For this test, saliva from six individuals are analyzed for any interference with the drugs or internal standards. The lower limit of quantification (LLOQ) is the lowest concentration of target analyte (in this case AMP and METH) that can be reliably quantified. This must be determined for each analyte and must have a response at least five times the signal of a blank sample. Carry over happens when remnants from a previous sample interfere with the next sample to be run. It is tested by running a blank sample immediately after a sample with high concentrations. If the blank has no interference greater than 20% of the lower limit of quantification, the method is considered clear of carryover.

The development of a calibration curve is one of the most important aspects of method validation. A curve is a graphical representation of the relationship between an analyte's concentration and the instrument's response. Each analyte must have it's own calibration curve of at least six samples of known concentration levels with 75% of the standards testing within 15% of the nominal value (20% in the case of the LLOQ). Another important aspect of the validation process is the evaluation of method accuracy. Within the EMA validation process, accuracy is described as the closeness of the determined value obtained by the method to the nominal concentration of the analyte. Accuracy is expressed in percentage and must be demonstrated within runs and between runs at four specific analyte concentration levels (LLOQ, low, medium, and high). Precision is described as the closeness of repeated measurements of the same sample. It must also be determined within run and between runs at the same analyte levels as accuracy. Dilution integrity testing simply ensures that the accuracy and precision of the samples is not affected by dilution and should be at similar dilutions that the actual samples undergo.

The background solution of the analyte is called the matrix. The matrix for this thesis is saliva. Matrix effect testing ensures that testing is not affected by differences in saliva. There must be six individual sources of saliva to which AMP, METH, and both internal standards will be added to (spiked with) and tested. Each analyte's response will be calculated as a matrix factor by comparing ratios of peak area with and without the saliva. The internal standard adjusted matrix factor should be less than 15%.

The final aspect of EMA method validation is the investigation of stability. Stability studies are needed to confirm that every step taken during sample preparation, analysis and storage do not affect the concentration of the analyte. Quality control samples (at three levels: low, high and stock concentrations) should be analyzed against a fresh calibration curve and the concentrations calculated. For this thesis, the conditions analyzed are: the initial concentrations, concentrations at room temperature up to 24 hours after (short-term stability), concentrations after a minimum of two freeze/thaw cycles (freeze/thaw stability), and concentrations after a single long-term freeze (long-term stability). This will be done for each analyte and each internal standard. All quality control stability samples should test within 15% of the nominal concentration to demonstrate stability. Once all of the sections above are complete and within acceptable standards, the validation will be complete. Because of the similarities of the EMA guidelines to the USFDA guidelines, it would not be too difficult to restructure the report should future USFDA approval be needed.

3 Experimental

This section gives the details of the conditions optimized and utilized in the final method.

3.1 Capillary Electrophoresis Instrumentation

A specially designed capillary electrophoresis instrument CE-FD (Capillary Electrophoresis – Fluorescence Detector) (Figure 6) was designed by the Analytical Department of TTU and made by the company OMEC, based out of Tartu, Estonia. [40] The instrument is a miniaturized and portable version of the full-sized CE instrumentation found in typical analytical labs. It is outfitted with a fluorescence detector, a power source and a safety power cut-off. It connects to a computer and purpose-built software using a standard USB cable. The instrument is smaller than most, more rugged, has considerably fewer moving parts and only takes a small amount of training for use. One of the objectives of the illegal drug team is to develop methods for testing common illegal drugs on this particular machine within a quick timeframe.

Figure 6. CE-FD instrument

3.2 Reagents and Samples

All reagents and standards were of analytical grade. Ultrapure water (Milli-Q) was provided by a Milli-Q integral water purification system. Standards of d,l-amphetamine (AMP) (1 mg mL^{-1} in methanol), d,l-methamphetamine (METH) (1 mg mL^{-1} in methanol) and powders were purchased from Lipomed AG (Switzerland). The internal standards of Benzylamine, BENZ (IS1) and Allocryptopine, ACP (IS2) are from Sigma-Aldrich (USA). Background electrolyte constituents, including sodium hydroxide, methanol and acetonitrile (ACN), phosphoric acid (85%), Tris(hydroxymethyl)aminomethane (TRIS), triethanolamine (TEA) were obtained from Sigma-Aldrich (USA).

Blank saliva samples were provided by seven volunteers, both male and female, aged 30-50. Volunteers submitted one sample via the Salivette® collection device tube (Sarstedt, Numbrecht, Germany) and approximately 3mL oral fluid in a collection tube. Each sample collected in the Salivette® collection device was assigned a sample reference number beginning with 2017W-. Reference numbers are associated only with the medical eligibility questionnaire of the donor while the consent forms were signed and collected on a separate sheet to assure donor anonymity. Donors were asked questions regarding recent illegal drug, prescription and supplement use to confirm their eligibility to serve as a "clean" sample. The donor volunteer questionnaire and blank consent form are attached in Appendix 3. Each donor sample was tested and confirmed to not have any interferences by processing and running each by itself and after inserting the internal standards.

Oral fluid samples were collected in Salivette® collection devices during a two-minute period. The Salivette® device was then centrifuged at 8000rpm for 10 min. The aqueous phase was discarded and 1.0mL acetonitrile was added to the swab in the Salivette® tube. The sample was then centrifuged an additional 2 min at 8000rpm. The centrifugate was then collected for testing. Each sample was spiked with a pre-prepared combined IS solution so that the final concentration in the sample was 300 ppm BENZ and 33.3 ppb ACP. Figure 7 shows the sample processing in order.

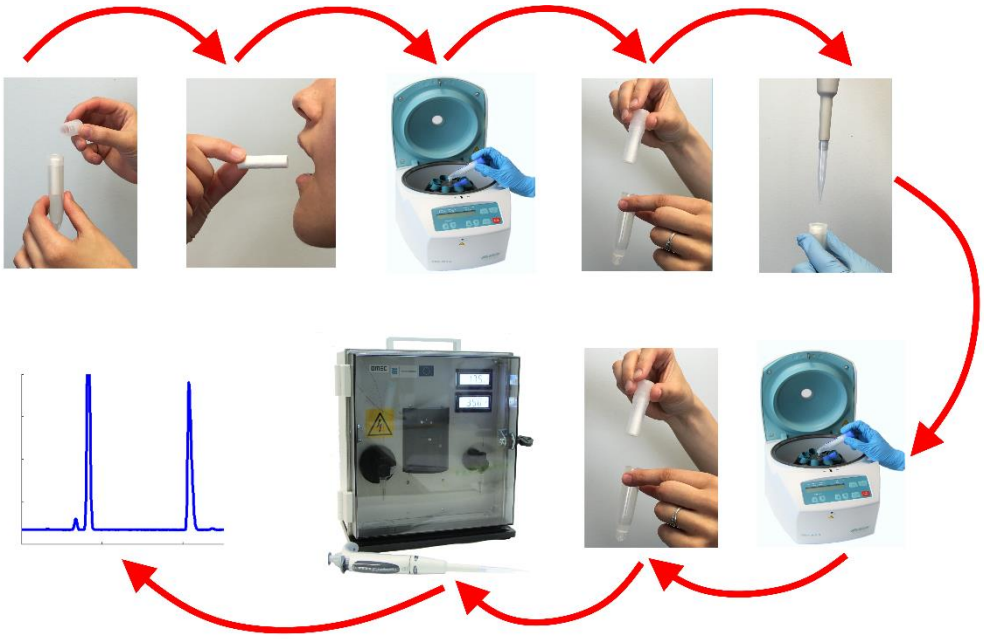

Figure 7. Visual of the sample processing steps

3.3 Method

Major stages of the method development process included:

- Research of current methods used for illegal drug determination, coordinate and work with other TTU researchers working on the illegal drug capillary electrophoresis program
- Research and test the best suited internal standard, ideally two standards for all samples
- Determine the best filter/emission wavelength range for the method to be run at
- Optimize method and sample preparation
- Optimize CE method for sample analysis
- Validate CE method according to the EMA recommendations
- Verify method on samples provided by partnership with the Estonian Police and are confirmed through currently used methods and instrumentation

The experiments were conducted on the portable CE-FD instrument using a fused silica coated capillary (i.d. 75 µm and o.d. 360 µm (Polymicro Technologies, Phoenix, AZ, USA) with an effective length of 40 cm and a total length of 58 cm. The new capillaries were conditioned using a 1.0 M NaOH solution and Milli-Q water. Each day the capillary was activated with a 10-minute wash of 1.0M NaOH and Milli-Q, followed by a 10

minute wash of the background electrolyte (BGE). Between every run, the capillary was washed for 2 minutes with the BGE. The instrument has five wavelength filter options, the fluorescence wavelength of 315 nm was chosen. Other instrument settings include a PMT voltage of 500V, 500 ms time resolution and a 10,000-individual count time-out. Samples were hydrodynamically injected at a height differential of 18 cm for 10 seconds. The voltage chosen during optimization was 20.0 kV with an applied current of approximately 50-65uA. The BGE chosen is a solution of 30 mM TRIS, 50mM H_3PO_4 in water, pH 2.5. Internal standard 1 (IS1) is BENZ at a concentration of 300 ppm in the final sample solution. Internal standard 2 (IS2) is ACP at a concentration of 33.3 ppb in the final sample solution.

Each of the factors chosen was tested during the optimization step of the method development. Table 2 lists each variable tested.

Table 2 Optimization Factors Tested

Factor	Values
Buffer Composition	TEA, TRIS
Buffer H3PO4 Concentration (mM)	50, 80, 100
Voltage (kV)	15, 18, 20, 21
Injection Time (s)	5, 10, 20, 30
Filter wavelength (nm)	315
Internal Standard 2 Identity	aminobenzoic acid, phenylalanine, tryptophan, tyrosine, benzylamine
Internal Standard 2 Concentration (ppm)	50, 100, 200, 300, 350, 500, 1000

4 Results and Discussion

The results in this section are presented in the order which the EMA guidelines are referenced in section 2.6.2 suggest for validation. There was however, a challenge in that there was an extremely limited amount of AMP and METH standard to work with. Due to the controlled nature of the substances and the cost, the lab is not expected to receive more until January 2018. Because of this, not all aspects of the EMA validation were able to be completed to the full standard and some of the secondary goals were not able to be accomplished. Areas in which the lack of available sample affected the outcome of the results are discussed in each section. Appendix 4 contains the area tables and additional information used for the calibration and calculations.

4.1 Selectivity, LLOQ and Carry Over

The EMA guidelines regarding selectivity state that 6 individual sources of blank matrix must be evaluated in order to prove there are no interferences from endogenous components of the matrix. In this case, seven volunteers provided blank saliva. That saliva was processed using the method described in Figure 6. Each blank sample was run as-is and with ISs added. See figures 8 and 9. The blank response of each was then compared to the LLOQ of the method for each IS, AMP, and METH. The LLOQ of the method can be calculated to be approximately five times the average noise. The average noise of blanks was a response of seven. Using the standard Signal-to-Noise ratio method, the signal must be 35 to meet the LLOQ guidelines. To qualify for selectivity, the blank response should be less than 20% of the response for AMP and METH and less than 5% of the response for the ISs. With the LLOQ of 35, the average blank response of seven for AMP and METH meets the less than 20% requirement. For the ISs, the average blank response of seven results in 3.2% of the average IS1 response from the LLOQ sample and 2.6% of the IS2 response. Therefore, both ISs, AMP and METH qualify for selectivity under the EMA guidelines.

Carry over happens when sample remains in the capillary after a high concentration has been run, despite the routine washing process. Each method should be evaluated for carry over by testing a blank sample immediately after testing a high concentration sample. There was no carry over observed for either AMP or METH after the highest concentration level available (500ppm) was tested.

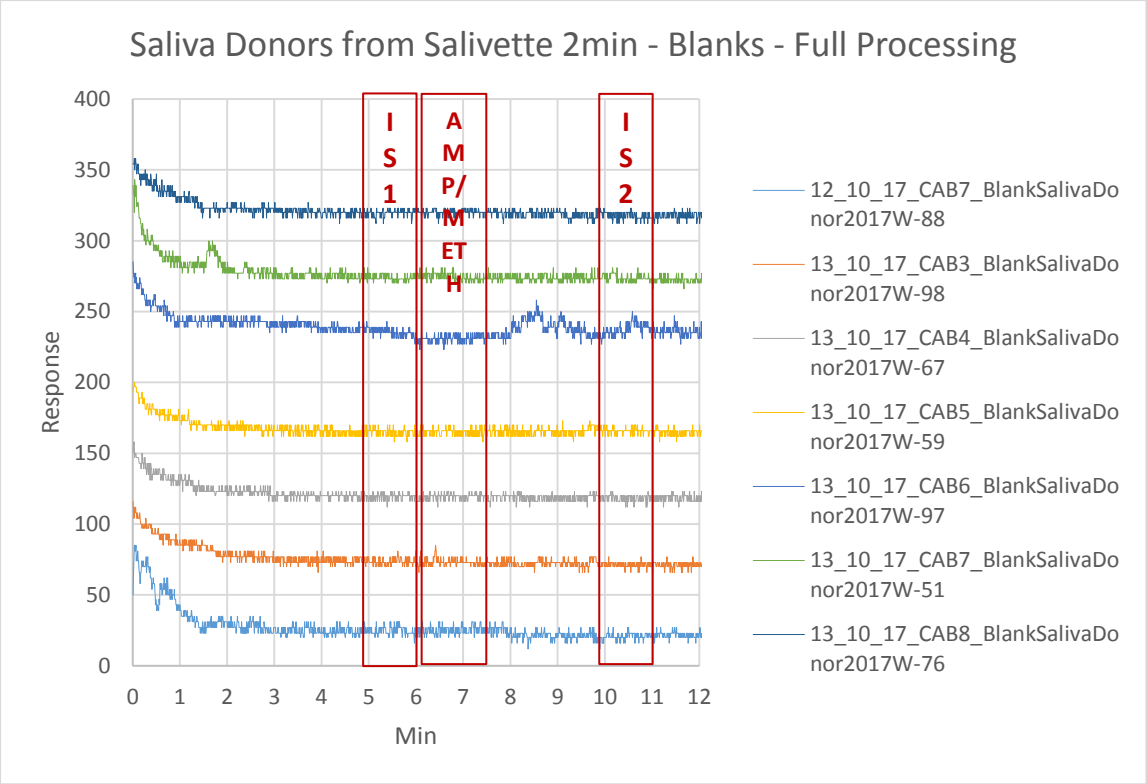

Figure 8. Saliva Donors from Salivette 2min - Blanks - Full Processing

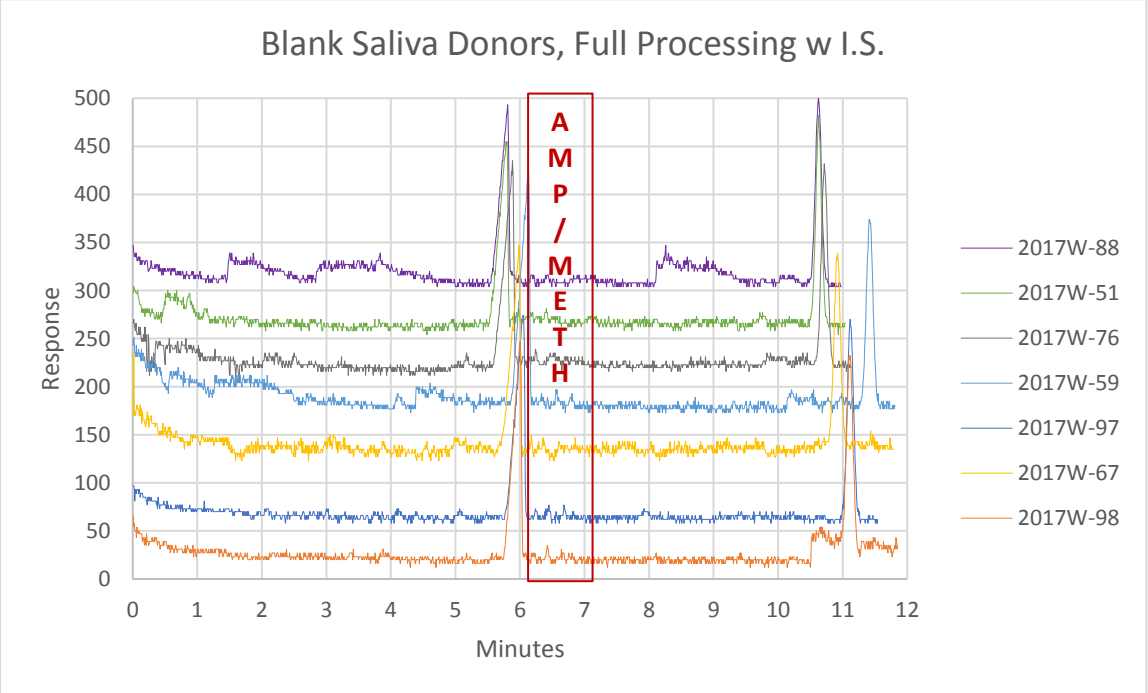

Figure 9. Blank Saliva Donors, Full Processing w I.S.

4.2 Calibration, Accuracy and Precision

The calibration curve is the most important part of the validation process. The curve represents the relationship between the concentration of the analyte and the instrument's response (area under the curve). The curve is built by graphing the concentration versus the average peak area of the analyte's replicates. Each curve has been corrected by comparing the area of the analyte to the ratio of areas of both of the ISs (IS1/IS2).

First, the instrumental settings were tested using low amounts of AMP and METH diluted in ACN. Figures 10 and 11 show the calibration curves from the ACN analysis. These tests and subsequent calibration curve allowed the instrumental detection and quantification limits to be calculated. At this point in the validation, the lack of sufficient AMP and METH to fully complete the validation became apparent to the researchers. Because of this, only one replicate of 95.2 ppm and 190.5 ppm AMP/METH samples were made to conserve sample for the final method analysis. This lead to a larger error and lower overall accuracy for these points, as is evident in the curves. However, since this curve was used only to determine the instrumental limits of detection and quantification and not the method limits, the research proceeded forward.

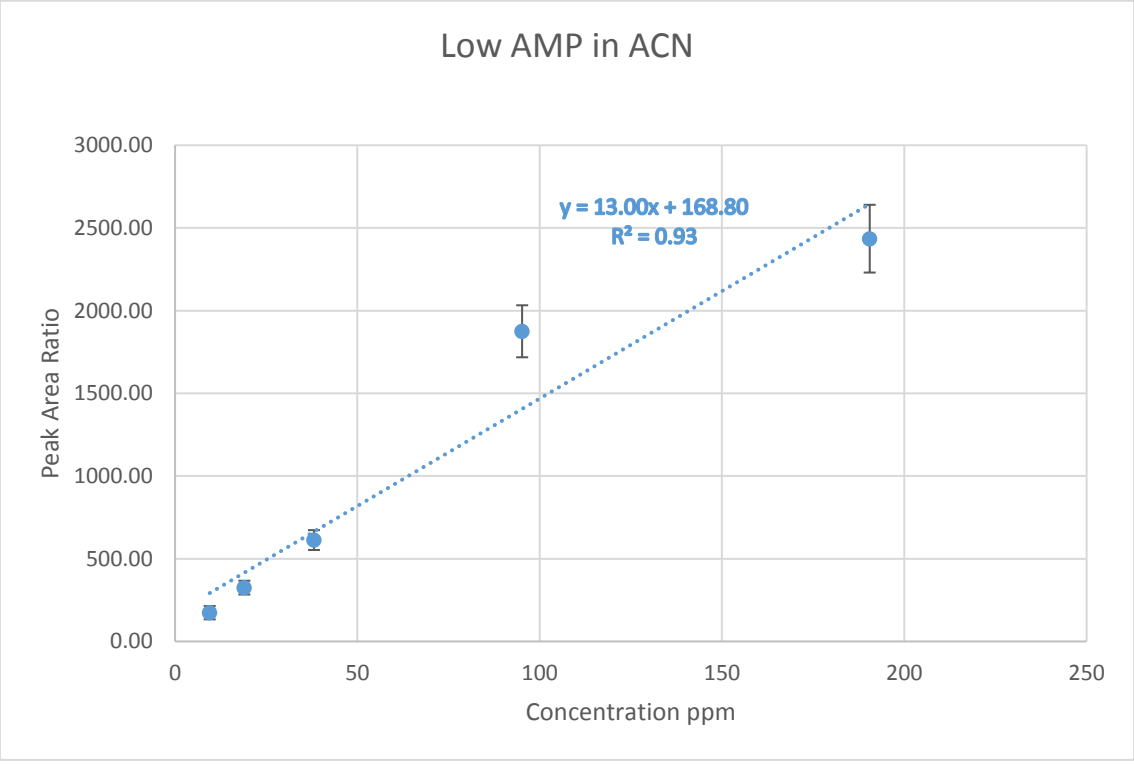

Figure 10. Low AMP in ACN

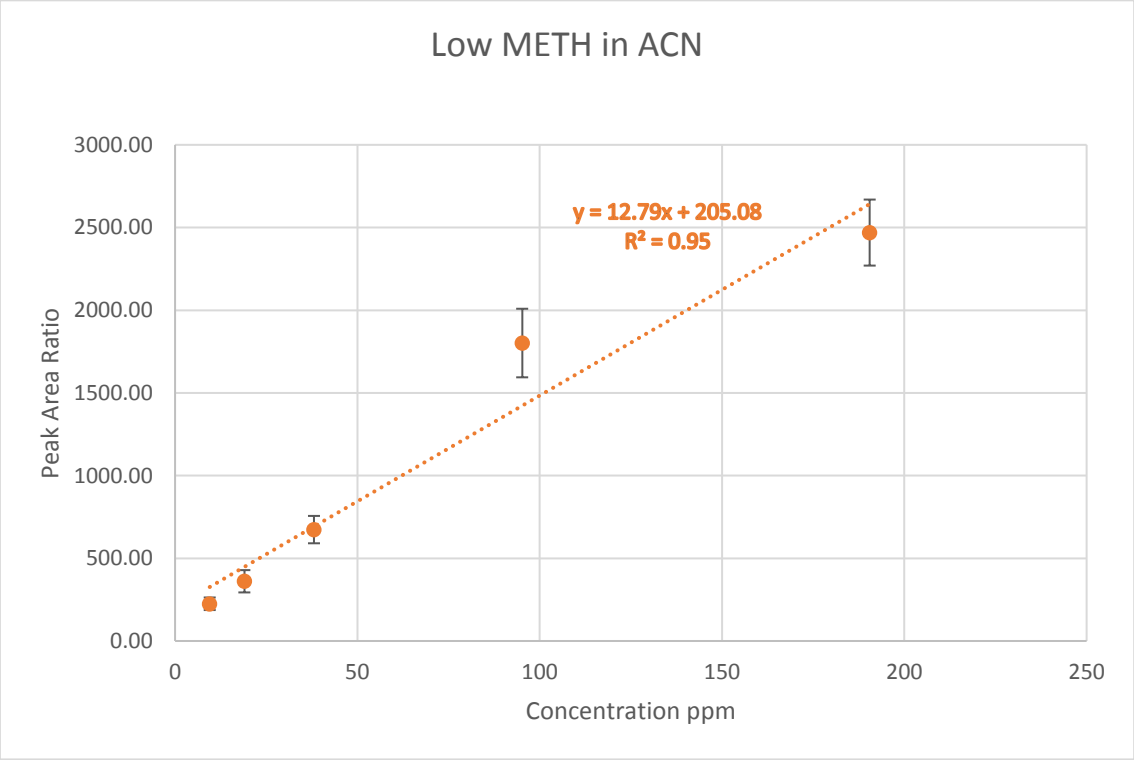

Figure 11. Low METH in ACN

The instrument limit of detection (ILOD) is the lowest quantity of a substance that can be distinguished from the absence of the same substance. It is different from the instrument level of quantification (ILOQ) in that although the instrument may be able to "see" the substance (ILOD) it cannot accurately quantify the substance at that level (ILOQ). Using the calculation ILOD = (3*SD)/slope, the ILOD of detection for AMP was calculated to be 9.3 ppm while the ILOD for METH was calculated at 8.9 ppm. This lines up with visual observation of the instrumental detection limit where 9.5 ppm was acceptable but 5 ppm was not. The ILOQ = (10*SD)/slope. Therefore, the ILOQ for AMP is 30.9 ppm and 29.7 ppm for METH.

Moving forward with the full method, a calibration curve was built using blank saliva samples that were spiked with known amounts of AMP and METH, processed according to the method and tested. Although the EMA guidelines require six points on the curve, only five points were included in this thesis. The original sixth point, intended to be at 50 ppm was too far below the acceptable signal to noise ratio to be included in the calibration. With the three low level standards (100, 150, 225 ppm) it was possible to include AMP

and METH in the standard solution together without risk to the matrix. But for the two highest levels (325, 500 ppm) AMP and METH were tested in separate standard solutions to allow for proper matrix effect since adding both into one sample would create a matrix of ACN without saliva. Figure 12 shows an electropherogram of each concentration from a single day calibration.

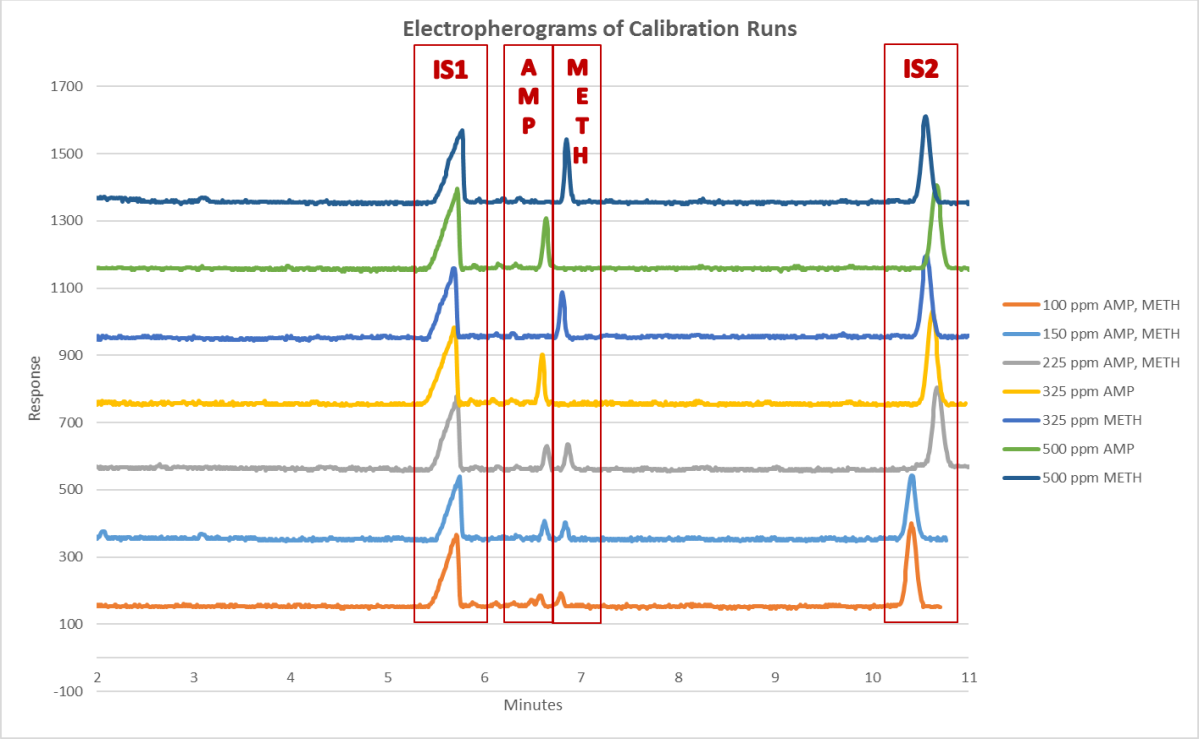

Figure 12. Electropherograms of Calibration Runs

The calibration was completed three days in a row for the inter-day precision and accuracy measurements and most of the samples also run for intra-day calculations as well. The main calibration curves below (Figures 13 and 14) are shown with the three inter-day calibrations combined and corrected using the IS ratio (IS1/IS2). Table 3 provides an overview of four calibrations for easy comparison.

Table 3. Overview of calibration information

Table 4. Overview of calibration information Analyte	Range, ppm	R^2	Equations	IDL, IQL*	LOD, LOQ**
Low Amphetamine, in ACN	9.5-190.5	0.93	Y = 13.00X + 168.80	9.3 ppm	30.9 ppm
Amphetamine	100-500	0.95	Y = 1.68X + 50.20		
Low Methamphetamine, in ACN	9.5-190.5	0.95	Y = 12.79X + 205.08	8.9 ppm	29.7 ppm
Methamphetamine	100-500	0.98	Y = 2.15X - 12.70		

*IDL, IQL – instrumental detection and quantification limits

**LOD, LOQ – limits of detection and quantification using full processing

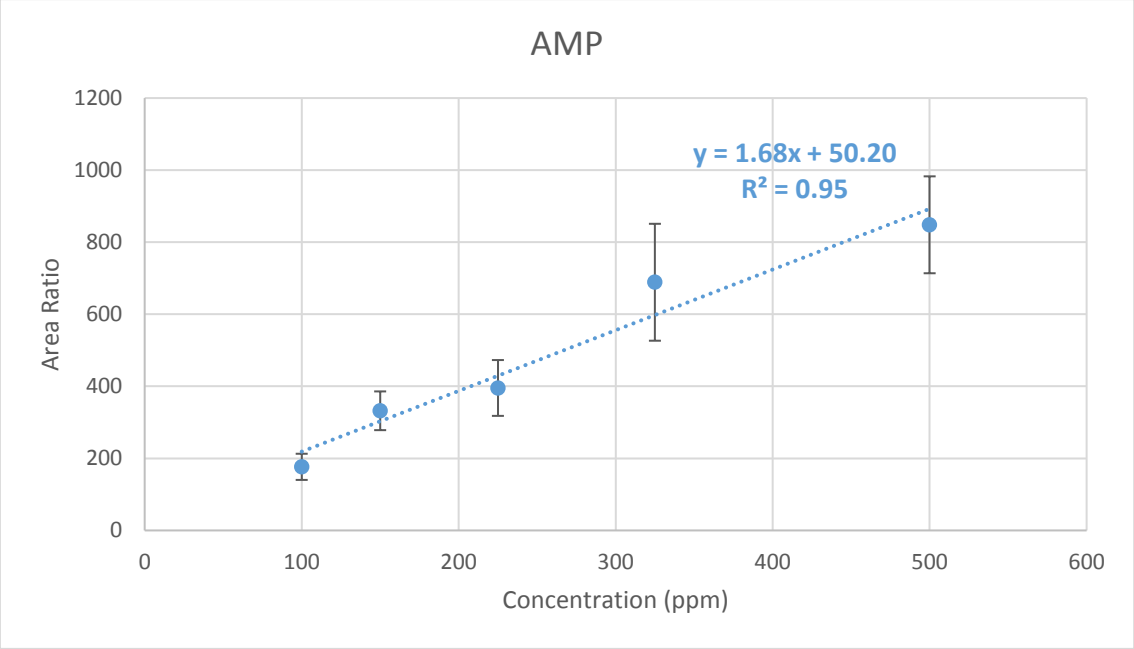

Figure 13. AMP calibration with full sample processing

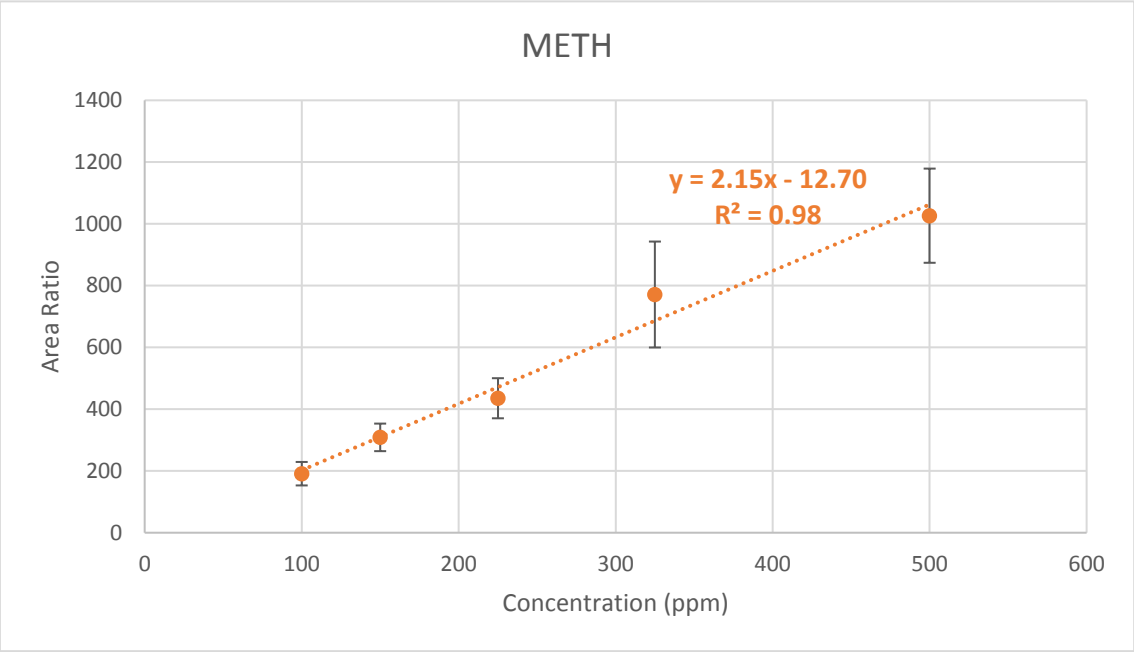

Figure 14. METH calibration with full sample processing

4.3 Dilution Integrity and Matrix Effect

Dilution integrity is tested by adding analyte above the highest level of the calibration curve, processing the sample with the method process, then diluting the sample back down to within the calibration. Because of the lack of AMP and METH, dilution integrity was not tested from above the calibration curve. To make 1 mL of spiked saliva sample above 500 ppm, more than 500 µL of AMP and METH each would have been needed and that amount was not available.

The matrix factor for AMP is calculated at 1.13 and the METH matrix factor is calculated to 0.99. The internal standard normalized matrix factors are calculated to be 1.35 and 1.19 for AMP and METH, respectively. Recovery on the method ranged from 17 to 23 % depending upon the concentration.

4.4 Stability

Stability of many of the solutions used in the method were established by others previous to this thesis. The buffer solution is stable at room temperature for months at a time. All saliva samples were stored in a cooler with ice packs until they reached the laboratory.

Once in the lab, the samples were processed, then stored in the lab freezer. All standards, including the internal standard solution and any containing AMP and METH were also stored in the freezer when not in use to prevent degradation in light or heat. Standards diluted in saliva and ACN were tested weeks afterwards with no major loss of peak area, demonstrating reasonable stability.

5 Weekend Music Festival Samples

During the summer, the illegal drug team at TTU collaborated with the Pärnu Police Department during the Weekend Music Festival to collect real world oral fluid samples from people suspected of being under the influence. The festival was held during the 4-5th of August, 2017 in Pärnu, Estonia. Although only specific members of the team were present during the sample collection and initial testing, the samples and results of the testing are available for the entire illegal drug testing team to utilize in their research.

In total, 37 samples were collected and analyzed for illegal drug abuse. This study presents the results of five positive samples. Table 4 presents the calculated concentrations of each drug in the oral fluid sample based upon the peak area and the calibration curves for each. These samples were all positive for AMP and MDMA with most also testing positive for MDA, although not always at the quantifiable level.

Table 5. Calculated concentrations of each of the five samples positive for AMP

Sample number	AMP, ppm	METH, ppm	MDA, ppb	MDMA, ppb	MDME, ppb	Cocaine, ppb	Cocaethylene, ppb
1	2234±753	ND	60 LOD>LOQ	581±77	ND	ND	ND
2	877±81	ND	778±10	16910±650	ND	ND	ND
6	2609±1305	ND	ND	267±84	ND	ND	ND
8	2598±174	ND	LOD>LOQ	96±1	ND	ND	ND
22	1396 ±67	ND	426±3	6186±232	ND	ND	ND

*ND: Not Detected

Figure 15 A – E shows the electropherograms of each of the five samples. Note that the scale of each electropherogram is different. The amount of IS2 in each is the same, however the scales were left at the appropriate level to demonstrate the variation in actual drug amounts in oral fluid. It is worth mentioning that these samples were tested during the Weekend Festival, before the identity of IS1 was established in the method. However, all of the other aspects of the optimization were completed at the time (those previously listed in Table 2 as well as sample processing details) so these were tested on effectively

the same method as the final method that was validated. This confirms that the method does work in real life situations, with real world samples. The results of the entire project are going to be submitted for publication in a peer reviewed journal by the illegal drug team at Tallinn University of Technology in 2018.

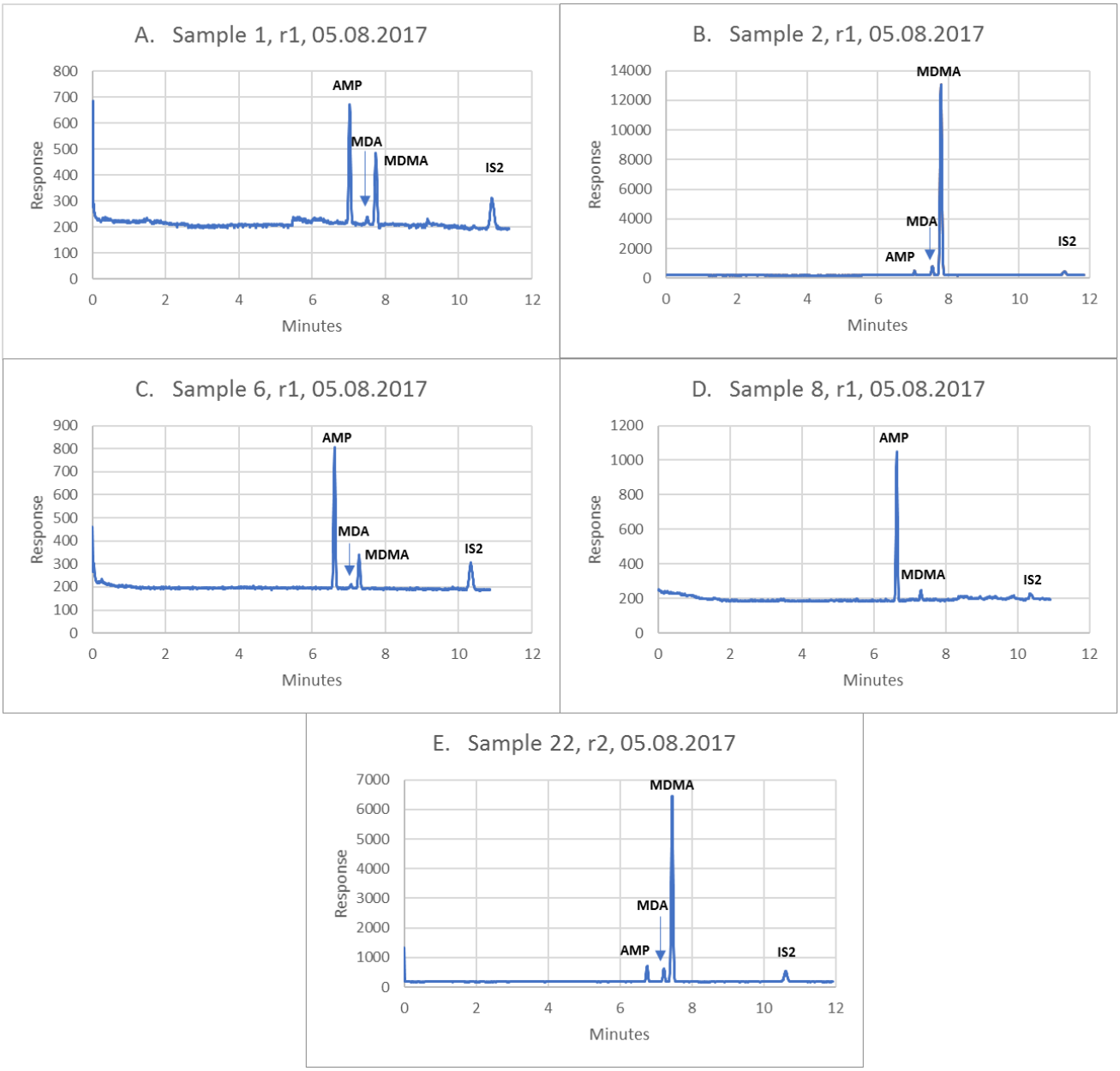

Figure 15A-E. Electropherograms of five samples positive for AMP from the Weekend Festival samples

6 Conclusion

The main aim of this thesis was to develop and validate a method that can rapidly and selectively quantify amphetamine and methamphetamine in a saliva sample using simple processing and TTU's novel portable capillary electrophoresis instrument. Much of the time spent was expended on developing and optimizing this method.

A method was developed, optimized and compared to the European Medicines Agency (EMA) guidelines on bioanalytical method validation. The final method involves testing on the patented portable CE (# EE01411U1) using a fused silica coated capillary (i.d. 75 μm and o.d. 360 μm with an effective length of 40 cm and a total length of 58 cm. The fluorescence detector was set at 315nm, with a voltage of 20.0 kV, and used a buffer solution comprised of 30 mM tris(hydroxymethyl)aminomethane (TRIS), 42.5 mM phosphoric acid (H_3PO_4). Sample injection was done for ten seconds hydrodynamically at a height of 18 cm. Most aspects of the validation were met although some allowances were made to account for a limited supply of pure AMP and METH standard to work with. Therefore, the hypothesis is confirmed.

While amphetamine and methamphetamine shortages made completing all aspects of the desired validation difficult, the method was designed to simultaneously test for cocaine, cocaethylene, MDA, MDEA, and MDMA. The lack of standards also prevented most of the secondary objectives from being reached. Further work is needed to finalize the validation, complete the swab efficiency comparison and prescription medication comparison once more standards become available.

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Appendix 1 – Transcript of e-mail to/from USFDA

FW: DrugInfo Comment Form FDA/CDER Site
CS
CDER SBIA <CDERSBIA@fda.hhs.gov>

Reply|
Fri 3/3/2017 8:49 PM
To:
'chelsagray@hotmail.com'
Dear Chelsa Brilla,

Thank you for writing to the Division of Drug Information, Small Business and Industry Assistance (SBIA), in the FDA's Center for Drug Evaluation and Research (CDER).

Please be advised that the guidance document entitled Guidance for Industry: Bioanalytical Method Validation, issued in September 2013, is the most current version available for this guidance which is still in "draft" format. We are unable to provide a timeframe on when a final version of this guidance document will be available. The FDA will announce in a Federal Register Notice the availability of any guidance that describes FDA's current thinking on a topic. You may search the Federal Register at https://www.federalregister.gov/agencies/food-and-drug-administration for current notices pertaining to publications of guidances issued by the FDA.

To review the FDA's policies and procedures for developing, issuing, and using guidance documents, please see Good Guidance Practices at 21CFR10.115. You may locate all official FDA Guidance Documents and other regulatory guidance in our database at http://www.fda.gov/RegulatoryInformation/Guidances/default.htm. This database allows you to conduct a search for guidance documents using key words, and you can narrow or filter your results by product, date issued, FDA organizational unit, type of document, subject, draft or final status, and comment period.

If in the future you have additional questions, please feel free to contact us again at CDERSBIA@fda.hhs.gov.

Register for CDER SBIA Regulatory Education for Industry (REdI): Generic Drugs Forum April 4-5, 2017
Review our Chronicles article "FDA Addresses Small Business Concerns with GDUFA II"

Best regards,

KDe
Drug Information Specialist
CDER Small Business and Industry Assistance
Division of Drug Information
Center for Drug Evaluation and Research
Food and Drug Administration

CDER has made it easier for small business to interact with the Agency via our Small Business Assistance and Industry Assistance contacts:
E-mail: CDERSBIA@fda.hhs.gov
Phone: (866).405.5367 or (301).796.6707
Website: http://www.fda.gov/CDERSBIA
CDER Small Business
survey: https://www.surveymonkey.com/s/CDERSBIA_Evaluation

For up-to-date drug information, follow the FDA's Division of Drug Information on Twitter @FDA_Drug_Info

This communication is consistent with 21 CFR 10.85(k) and constitutes an informal communication that represents our best judgment at this time but does not constitute an advisory opinion, does not necessarily represent the formal position of the FDA, and does not bind or otherwise obligate or commit the agency to the views expressed.

-----Original Message-----
From: druginfo@fda.hhs.gov [mailto:druginfo@fda.hhs.gov]
Sent: Thursday, March 02, 2017 4:08 AM
To: CDER DRUG INFO
Subject: DrugInfo Comment Form FDA/CDER Site

Name: Chelsa Brilla

E-Mail: chelsagray@hotmail.com

Comments: Hello,
I am looking for the most up-to-date guidance document on Bioanalytical Method Validation. The website includes the final document from 2001 and a draft document labeled Bioanalytical Method Validation [Revised Final] from 2013. Is there a final approved document from the 2013 draft? Is so, could you please email a copy to me? Also, if there is an updated document, please know that it is not showing up in search results on the website. Any assistance with this would be appreciated.
Thank you.

URL:

Appendix 2 – Design of Experiment

Each X represents a sample needed to complete the EMA validation in entirety:

Samples Needed						
Type of Sample	**Selectivity .**	**Calibration .**	**Accuracy Precision**	**Dilution .**	**Matrix .**	**Stability .**
Saliva 1	X				XX	
Saliva 2	X				XX	
Saliva 3	X				XX	
Saliva 4	X				XX	
Saliva 5	X				XX	
Saliva 6	X				XX	
Blank		XX				
Zero w/I.S.s		XXXXXX				
LLOQ		XXXXXX	XXXXX			
Level 2		XXXXXX				
Level 3		XXXXXX				
Level 4		XXXXXX				
Level 5		XXXXXX				
ULOQ		XXXXXX				
Low QC			XXXXX			XXX
Mid QC			XXXXX			
High QC			XXXXX			XXX
Dilution 1				XXXXX		
Dilution 2				XXXXX		
Dilution 3				XXXXX		
ACN Matrix					XX	
BA Stock						X
AMP Stock						X
METH Stock						X
ACP Stock						X

Appendix 3 – Blank Donor Questionnaire and Consent Form

Page 1

Questionnaire and Consent Form for Saliva Donation to TUT Illegal Drug Project

Age:_____Gender:_____Sample ID:_____

Have you used illegal drugs in the past 6 months?_____

List prescription and OTC meds used in the past month:_____

_____

_____

_____

_____

List of any additional supplements used in the past month (vitamins, workout enhancers):_____

_____

_____

_____

Are you taking medication for Narcolepsy or ADHD? _____

_____

Have you used Sudafed or cold medications in the past month? If so, what?_____

_____

_____

Have you used weight loss supplements in the past month?_____

_____

Page 2

Questionnaire and Consent Form for Saliva Donation to TUT Illegal Drug Project

Release:

I am freely donating my saliva for scientific research. I understand that it will be used in method development and validation for an illegal drug screen. I understand that the results and data will be published in a master's thesis that is available to the public and may be submitted for journal publication. My personal identifying information and health history WILL NOT be published or shared in any way and will be destroyed upon successful defense of the thesis in January 2018.

Printed Name Signature Date

Appendix 4 – Additional Data

Full Calibration Data, Saliva, Full Sample Processing – Area under curves

Level	Date	Replicate	Run	IS1 - Benz Area	Amp Area	Meth Area	IS2 - Allo Area	IS1/IS2 Ratio	Conc ppm
1	**11/5/2017**	1	1	3159	125.2	144.7	2461	1.28	100
1	**11/5/2017**	2	1	3573	208.5	214.3	3048	1.17	100
1	**11/5/2017**	3	1	3427	169.1	187.1	2873	1.19	100
1	**11/6/2017**	1	1	3901	215.9	256.9	3282	1.19	100
1	**11/6/2017**	2	1	3827	226.7	245.5	3488	1.10	100
1	**11/6/2017**	3	1	4394	235.8	255.7	3765	1.17	100
1	**11/7/2017**	1	1	3346	200.5	207.2	2917	1.15	100
1	**11/7/2017**	2	1	3914	220.8	221.8	3489	1.12	100
1	**11/7/2017**	3	1	5034	265.8	291.9	3972	1.27	100
AVG				3842	207.6	225.0	3255	1.18	100
SD				580	40.5	43.6	477	0.06	100
%CV				15	19.5	19.4	15	5.19	100
2	**11/8/2017**	1	1	4184	489.8	454.4	3590	1.17	150
2	**11/8/2017**	2	3	3620	360.9	347.8	2986	1.21	150
2	**11/8/2017**	3	1	3760	447.2	383.0	3208	1.17	150
2	**11/6/2017**	1	1	3482	367.0	352.9	2747	1.27	150
2	**11/6/2017**	2	1	3600	380.0	366.2	2682	1.34	150
2	**11/6/2017**	3	1	3090	330.2	306.8	2499	1.24	150
2	**11/7/2017**	1	1	3554	435.6	405.7	3152	1.13	150
2	**11/7/2017**	2	1	3212	397.4	356.1	2782	1.15	150
2	**11/7/2017**	3	1	3347	418.0	401.5	2556	1.31	150
AVG				3539	402.9	374.9	2911	1.22	150
SD				321	49.6	42.3	355	0.07	150
%CV				9	12.3	11.3	12	6.05	150
3	**11/5/2017**	1	1	3194	374.6	452.4	2662	1.20	225
3	**11/5/2017**	2	1	3545	435.7	484.7	2626	1.35	225
3	**11/5/2017**	3	1	3709	426.1	459.0	--	--	225
3	**11/5/2017**	3	2	3511	417.8	459.5	2895	1.21	225
3	**11/6/2017**	1	1	3990	470.9	520.1	3277	1.22	225
3	**11/6/2017**	2	1	3540	455.9	497.1	2970	1.19	225
3	**11/6/2017**	3	1	4675	499.0	537.0	3939	1.19	225
3	**11/7/2017**	1	1	4988	658.7	661.8	4306	1.16	225
3	**11/7/2017**	2	1	4038	514.1	542.1	3468	1.16	225
3	**11/7/2017**	3	1	4619	443.2	548.2	3959	1.17	225
AVG				3981	469.6	516.2	3345	1.21	225

Level	Date	Replicate	Run	IS1 - Benz Area	Amp Area	Meth Area	IS2 - Allo Area	IS1/IS2 Ratio	Conc ppm
SD				597	77.6	62.6	612	0.06	225
%CV				15	16.5	12.1	18	4.83	225
4 Amp	**11/5/2017**	1	1	3961	755.0		2890	1.37	325
4 Amp	**11/5/2017**	2	1	3492	778.0		2692	1.30	325
4 Amp	**11/5/2017**	3	1	3735	661.9		3085	1.21	325
4 Amp	**11/6/2017**	1	1	3780	781.9		3117	1.21	325
4 Amp	**11/6/2017**	2	1	4493	1032.7		3938	1.14	325
4 Amp	**11/6/2017**	3	1	4294	773.2		3478	1.23	325
4 Amp	**11/7/2017**	1	1	4082	875.8		2554	1.60	325
4 Amp	**11/7/2017**	2	1	4102	981.9		3402	1.21	325
4 Amp	**11/7/2017**	3	1	5792	1142.5		4882	1.19	325
Avg				4192	864.8		3338	1.27	325
SD				671	156.2		717	0.14	325
%CV				16	18.1		21	10.90	325
4 Meth	**11/5/2017**	1	1	3162		735.1	2636	1.20	325
4 Meth	**11/5/2017**	2	1	3149		732.2	2569	1.23	325
4 Meth	**11/5/2017**	3	1	3443		715.3	2766	1.24	325
4 Meth	**11/6/2017**	1	1	3821		961.6	3402	1.12	325
4 Meth	**11/6/2017**	2	1	4065		833.1	3173	1.28	325
4 Meth	**11/6/2017**	3	1	4759		966.8	3846	1.24	325
4 Meth	**11/7/2017**	1	1	5084		1153.1	4413	1.15	325
4 Meth	**11/7/2017**	2	1	4452		993.6	3810	1.17	325
4 Meth	**11/7/2017**	3	1	5223		1187.6	4473	1.17	325
Avg				4129		919.8	3454	1.20	325
SD				796		178.3	728	0.05	325
%CV				19		19.4	21	4.26	325
5 Amp	**11/5/2017**	1	1	2962	768.3		2531	1.17	500
5 Amp	**11/5/2017**	2	1	3449	922.2		2902	1.19	500
5 Amp	**11/5/2017**	3	1	3747	890.2		3114	1.20	500
5 Amp	**11/6/2017**	1	1	4712	1160.0		3513	1.34	500
5 Amp	**11/6/2017**	2	1	3964	1036.9		3449	1.15	500
5 Amp	**11/6/2017**	3	1	3747	885.8		3137	1.19	500
5 Amp	**11/7/2017**	1	1	4906	1218.5		3863	1.27	500
5 Amp	**11/7/2017**	2	1	4443	1264.9		3851	1.15	500
5 Amp	**11/7/2017**	3	1	4378	1044.5		3761	1.16	500
Avg				4034	1021.3		3347	1.20	500
SD				629	168.7		460	0.06	500
%CV				16	16.5		14	5.24	500

Level	Date	Replicate	Run	IS1 - Benz Area	Amp Area	Meth Area	IS2 - Allo Area	IS1/IS2 Ratio	Conc ppm
5 Meth	11/5/2017	1	1	2760		987.9	2374	1.16	500
5 Meth	11/5/2017	2	1	3477		1040.7	2684	1.30	500
5 Meth	11/5/2017	3	1	4085		1112.9	3286	1.24	500
5 Meth	11/6/2017	1	1	3386		1184.8	2857	1.18	500
5 Meth	11/6/2017	2	1	4211		1387.7	3378	1.25	500
5 Meth	11/6/2017	3	1	4386		1274.9	3771	1.16	500
5 Meth	11/7/2017	1	1	3933		1305.2	3322	1.18	500
5 Meth	11/7/2017	2	1	4158		1316.9	3474	1.20	500
5 Meth	11/7/2017	3	1	5937		1640.3	4626	1.28	500
Avg				4037		1250.1	3308	1.22	500
SD				877		198.4	657	0.05	500
%CV				22		15.9	20	4.16	500

Full Calibration Data, Saliva, Full Sample Processing – Peak Area Ratios

Level	Date	Conc ppm	Amp/IS 1	Meth/IS 1	Amp/IS 2	Meth/IS 2	Amp/(IS1/IS2)	Meth/(IS1/IS2)
1	11/5/2017	100	0.04	0.05	0.05	0.06	97.53	112.73
1	11/5/2017	100	0.06	0.06	0.07	0.07	177.82	182.81
1	11/5/2017	100	0.05	0.05	0.06	0.07	141.73	156.83
1	11/6/2017	100	0.06	0.07	0.07	0.08	181.63	216.12
1	11/6/2017	100	0.06	0.06	0.07	0.07	206.66	223.78
1	11/6/2017	100	0.05	0.06	0.06	0.07	202.06	219.09
1	11/7/2017	100	0.06	0.06	0.07	0.07	174.80	180.57
1	11/7/2017	100	0.06	0.06	0.06	0.06	196.83	197.72
1	11/7/2017	100	0.05	0.06	0.07	0.07	209.67	230.29
AVG		100	0.05	0.06	0.06	0.07	176.53	191.11
SD		100	0.01	0.01	0.01	0.01	36.32	38.00
%CV		100	11.72	10.14	8.87	8.36	20.57	19.89
2	11/8/2017	150	0.12	0.11	0.14	0.13	420.35	389.98
2	11/8/2017	150	0.10	0.10	0.12	0.12	297.64	286.89
2	11/8/2017	150	0.12	0.10	0.14	0.12	381.56	326.79
2	11/6/2017	150	0.11	0.10	0.13	0.13	289.53	278.39
2	11/6/2017	150	0.11	0.10	0.14	0.14	283.13	272.82
2	11/6/2017	150	0.11	0.10	0.13	0.12	266.98	248.05
2	11/7/2017	150	0.12	0.11	0.14	0.13	386.35	359.80
2	11/7/2017	150	0.12	0.11	0.14	0.13	344.16	308.45
2	11/7/2017	150	0.12	0.12	0.16	0.16	319.24	306.64

Level	Date	Conc ppm	Amp/IS 1	Meth/IS 1	Amp/IS 2	Meth/IS 2	Amp/(IS1/IS2)	Meth/(IS1/IS2)
AVG		150	0.11	0.11	0.14	0.13	332.10	308.65
SD		150	0.01	0.01	0.01	0.01	53.77	44.60
%CV		150	8.34	7.40	8.20	9.23	16.19	14.45
3	**11/5/2017**	225	0.12	0.14	0.14	0.17	312.20	376.98
3	**11/5/2017**	225	0.12	0.14	0.17	0.18	322.74	359.00
3	**11/5/2017**	225	0.11	0.12	--	--	--	--
3	**11/5/2017**	225	0.12	0.13	0.14	0.16	344.44	378.85
3	**11/6/2017**	225	0.12	0.13	0.14	0.16	386.65	427.09
3	**11/6/2017**	225	0.13	0.14	0.15	0.17	382.56	417.15
3	**11/6/2017**	225	0.11	0.11	0.13	0.14	420.40	452.38
3	**11/7/2017**	225	0.13	0.13	0.15	0.15	568.59	571.28
3	**11/7/2017**	225	0.13	0.13	0.15	0.16	441.52	465.57
3	**11/7/2017**	225	0.10	0.12	0.11	0.14	379.88	469.87
AVG		225	0.12	0.13	0.14	0.16	395.44	435.35
SD		225	0.01	0.01	0.02	0.02	77.48	64.90
%CV		225	9.12	6.81	11.05	9.51	19.59	14.91
4 Amp	**11/5/2017**	325	0.19		0.26		550.80	
4 Amp	**11/5/2017**	325	0.22		0.29		599.79	
4 Amp	**11/5/2017**	325	0.18		0.21		546.82	
4 Amp	**11/6/2017**	325	0.21		0.25		644.70	
4 Amp	**11/6/2017**	325	0.23		0.26		905.16	
4 Amp	**11/6/2017**	325	0.18		0.22		626.13	
4 Amp	**11/7/2017**	325	0.21		0.34		548.08	
4 Amp	**11/7/2017**	325	0.24		0.29		814.33	
4 Amp	**11/7/2017**	325	0.20		0.23		963.02	
Avg		325	0.21		0.26		688.76	
SD		325	0.02		0.04		162.23	
%CV		325	10.62		15.16		23.55	
4 Meth	**11/5/2017**	325		0.23		0.28		612.80
4 Meth	**11/5/2017**	325		0.23		0.28		597.50
4 Meth	**11/5/2017**	325		0.21		0.26		574.68
4 Meth	**11/6/2017**	325		0.25		0.28		855.98
4 Meth	**11/6/2017**	325		0.20		0.26		650.16
4 Meth	**11/6/2017**	325		0.20		0.25		781.24
4 Meth	**11/7/2017**	325		0.23		0.26		1000.86
4 Meth	**11/7/2017**	325		0.22		0.26		850.31
4 Meth	**11/7/2017**	325		0.23		0.27		1017.11

Level	Date	Conc ppm	Amp/IS 1	Meth/IS 1	Amp/IS 2	Meth/IS 2	Amp/(IS1/IS2)	Meth/(IS1/IS2)
Avg		325		0.22		0.27		771.18
SD		325		0.02		0.01		171.50
%CV		325		7.05		4.42		22.24
5 Amp	**11/5/2017**	500	0.26		0.30		656.61	
5 Amp	**11/5/2017**	500	0.27		0.32		775.93	
5 Amp	**11/5/2017**	500	0.24		0.29		739.84	
5 Amp	**11/6/2017**	500	0.25		0.33		864.77	
5 Amp	**11/6/2017**	500	0.26		0.30		902.17	
5 Amp	**11/6/2017**	500	0.24		0.28		741.66	
5 Amp	**11/7/2017**	500	0.25		0.32		959.58	
5 Amp	**11/7/2017**	500	0.28		0.33		1096.41	
5 Amp	**11/7/2017**	500	0.24		0.28		897.15	
Avg		500	0.25		0.30		848.24	
SD		500	0.02		0.02		134.45	
%CV		500	6.41		6.46		15.85	
5 Meth	**11/5/2017**	500		0.36		0.42		849.70
5 Meth	**11/5/2017**	500		0.30		0.39		803.12
5 Meth	**11/5/2017**	500		0.27		0.34		895.12
5 Meth	**11/6/2017**	500		0.35		0.41		999.88
5 Meth	**11/6/2017**	500		0.33		0.41		1113.20
5 Meth	**11/6/2017**	500		0.29		0.34		1096.07
5 Meth	**11/7/2017**	500		0.33		0.39		1102.42
5 Meth	**11/7/2017**	500		0.32		0.38		1100.34
5 Meth	**11/7/2017**	500		0.28		0.35		1278.13
Avg		500		0.31		0.38		1026.44
SD		500		0.03		0.03		152.49
%CV		500		9.88		8.17		14.86

Full Calibration Data, Saliva, Full Sample Processing – Back Calculation of Standard (Samples in Red do not meet the accuracy/precision minimum of the EMA guideline)

Level	Date	Conc ppm	Amp BackCalc ppm	% of Actual	Meth BackCalc ppm	% of Actual
1	**11/5/2017**	100	28.09	28.09	58.31	58.31
1	**11/5/2017**	100	75.75	75.75	90.90	90.90
1	**11/5/2017**	100	54.33	54.33	78.82	78.82
1	**11/6/2017**	100	78.01	78.01	106.38	106.38
1	**11/6/2017**	100	92.87	92.87	109.95	109.95
1	**11/6/2017**	100	90.14	90.14	107.76	107.76
1	**11/7/2017**	100	73.96	73.96	89.86	89.86
1	**11/7/2017**	100	87.04	87.04	97.83	97.83
1	**11/7/2017**	100	94.66	94.66	112.97	112.97
AVG		100	74.98	74.98	94.75	94.75
SD		100	21.56	21.56	17.67	17.67
%CV		100	28.75	28.75	18.65	18.65
2	**11/8/2017**	150	219.71	146.47	187.22	124.81
2	**11/8/2017**	150	146.87	97.92	139.28	92.86
2	**11/8/2017**	150	196.69	131.12	157.84	105.23
2	**11/6/2017**	150	142.06	94.71	135.33	90.22
2	**11/6/2017**	150	138.26	92.17	132.74	88.50
2	**11/6/2017**	150	128.68	85.78	121.23	80.82
2	**11/7/2017**	150	199.53	133.02	173.18	115.46
2	**11/7/2017**	150	174.49	116.33	149.31	99.54
2	**11/7/2017**	150	159.69	106.46	148.47	98.98
AVG		150	167.33	111.55	149.40	99.60
SD		150	31.91	21.28	20.73	13.82
%CV		150	19.07	12.71	13.88	9.25
3	**11/5/2017**	225	155.52	69.12	181.17	80.52
3	**11/5/2017**	225	161.77	71.90	172.81	76.81
3	**11/5/2017**	225	--	--	--	--
3	**11/5/2017**	225	174.65	77.62	182.04	80.91
3	**11/6/2017**	225	199.71	88.76	204.47	90.87
3	**11/6/2017**	225	197.28	87.68	199.85	88.82
3	**11/6/2017**	225	219.74	97.66	216.23	96.10
3	**11/7/2017**	225	307.71	136.76	271.50	120.67
3	**11/7/2017**	225	232.28	103.23	222.36	98.83
3	**11/7/2017**	225	195.69	86.97	224.35	99.71
AVG		225	204.93	91.08	208.31	92.58

Level	Date	Conc ppm	Amp BackCalc ppm	% of Actual	Meth BackCalc ppm	% of Actual
SD		225	45.99	20.44	30.17	13.41
%CV		225	22.44	9.97	14.49	6.44
4 Amp	**11/5/2017**	325	297.14	91.43		
4 Amp	**11/5/2017**	325	326.23	100.38		
4 Amp	**11/5/2017**	325	294.78	90.70		
4 Amp	**11/6/2017**	325	352.88	108.58		
4 Amp	**11/6/2017**	325	507.48	156.15		
4 Amp	**11/6/2017**	325	341.86	105.19		
4 Amp	**11/7/2017**	325	295.53	90.93		
4 Amp	**11/7/2017**	325	453.57	139.56		
4 Amp	**11/7/2017**	325	541.83	166.72		
Avg		325	379.03	116.63		
SD		325	96.30	29.63		
%CV		325	25.41	7.82		
4 Meth	**11/5/2017**	325			290.81	89.48
4 Meth	**11/5/2017**	325			283.70	87.29
4 Meth	**11/5/2017**	325			273.09	84.03
4 Meth	**11/6/2017**	325			403.87	124.27
4 Meth	**11/6/2017**	325			308.18	94.82
4 Meth	**11/6/2017**	325			369.12	113.58
4 Meth	**11/7/2017**	325			471.23	144.99
4 Meth	**11/7/2017**	325			401.23	123.46
4 Meth	**11/7/2017**	325			478.78	147.32
Avg		325			364.44	112.14
SD		325			79.73	24.53
%CV		325			21.88	6.73
5 Amp	**11/5/2017**	500	359.95	71.99		
5 Amp	**11/5/2017**	500	430.78	86.16		
5 Amp	**11/5/2017**	500	409.35	81.87		
5 Amp	**11/6/2017**	500	483.51	96.70		
5 Amp	**11/6/2017**	500	505.71	101.14		
5 Amp	**11/6/2017**	500	410.44	82.09		
5 Amp	**11/7/2017**	500	539.79	107.96		
5 Amp	**11/7/2017**	500	621.00	124.20		
5 Amp	**11/7/2017**	500	502.73	100.55		
Avg		500	473.70	94.74		
SD		500	79.81	15.96		
%CV		500	16.85	3.37		

Level	Date	Conc ppm	Amp BackCalc ppm	% of Actual	Meth BackCalc ppm	% of Actual
5 Meth	**11/5/2017**	500			400.95	80.19
5 Meth	**11/5/2017**	500			379.29	75.86
5 Meth	**11/5/2017**	500			422.06	84.41
5 Meth	**11/6/2017**	500			470.77	94.15
5 Meth	**11/6/2017**	500			523.45	104.69
5 Meth	**11/6/2017**	500			515.49	103.10
5 Meth	**11/7/2017**	500			518.44	103.69
5 Meth	**11/7/2017**	500			517.48	103.50
5 Meth	**11/7/2017**	500			600.13	120.03
Avg		500			483.12	96.62
SD		500			70.89	14.18
%CV		500			14.67	2.93

ACN Calibration Data, No Processing – Area under Curves

Level	Replicate	Run	IS1 - Benz	Amp	Meth	IS2 - Allo	IS1/IS2	Conc ppm
2	1	1	5016.33	144.53	192.66	5077.85	0.99	9.50
2	1	2	3481.60	130.93	165.66	3874.14	0.90	9.50
2	2	1	3973.55	218.25	218.36	4117.21	0.97	9.50
2	2	2	3963.26	196.98	204.26	4255.83	0.93	9.50
2	3	1	4182.18	122.58	282.79	4326.40	0.97	9.50
2	3	2	4477.61	173.84	218.64	4778.66	0.94	9.50
AVG			4182.42	164.52	213.73	4405.02	0.95	9.50
SD			522.56	38.25	39.14	443.94	0.03	9.50
%CV			12.49	23.25	18.31	10.08	3.36	9.50
3	1	1	4675.90	363.36	289.82	4544.18	1.03	19.00
3	1	2	3240.18	209.02	266.23	4130.92	0.78	19.00
3	2	3	4007.46	324.02	364.15	4285.30	0.94	19.00
3	2	2	3818.46	347.77	436.87	4160.03	0.92	19.00
3	3	1	3836.09	290.11	313.02	3881.11	0.99	19.00
3	3	2	4597.83	305.81	354.39	4721.96	0.97	19.00
AVG			4029.32	306.68	337.41	4287.25	0.94	19.00
SD			537.83	54.80	61.35	303.50	0.08	19.00
%CV			13.35	17.87	18.18	7.08	9.06	19.00
4	1	1	4483.14	615.34	697.60	4437.22	1.01	38.10
4	1	2	4123.90	562.18	511.56	4332.34	0.95	38.10
4	2	1	4037.65	599.41	636.98	3884.92	1.04	38.10

Level	Replicate	Run	IS1 - Benz	Amp	Meth	IS2 - Allo	IS1/IS2	Conc ppm
4	2	2	3857.42	591.67	700.49	4165.70	0.93	38.10
4	3	1	3780.16	511.02	670.08	4031.12	0.94	38.10
4	3	2	3271.51	562.28	571.75	4176.33	0.78	38.10
AVG			3925.63	573.65	631.41	4171.27	0.94	38.10
SD			404.07	37.17	75.63	199.18	0.09	38.10
%CV			10.29	6.48	11.98	4.78	9.46	38.10
5	1	1	4384.04	1751.46	1643.17	4415.56	0.99	95.20
5	1	2	4215.35	1896.59	1860.05	4415.16	0.95	95.20
Avg			4299.70	1824.03	1751.61	4415.36	0.97	95.20
SD			119.28	102.62	153.36	0.28	0.03	95.20
%CV			2.77	5.63	8.76	0.01	2.77	95.20
6	1	1	3441.33	2784.00	2816.99	3189.19	1.08	190.50
6	1	2	3334.74	2507.52	2549.15	3045.95	1.09	190.50
Avg			3388.04	2645.76	2683.07	3117.57	1.09	190.50
SD			75.37	195.50	189.39	101.29	0.01	190.50
%CV			2.22	7.39	7.06	3.25	1.02	190.50

ACN Calibration Data, No Processing – Peak Ratios

Level	Replicate	Run	Conc ppm	Amp/ IS1	Meth /IS1	Amp/ IS2	Meth /IS2	Amp/(IS1 /IS2)	Meth/(IS1/ IS2)
2	1	1	9.50	0.03	0.04	0.03	0.04	146.30	195.02
2	1	2	9.50	0.04	0.05	0.03	0.04	145.69	184.34
2	2	1	9.50	0.05	0.05	0.05	0.05	226.14	226.25
2	2	2	9.50	0.05	0.05	0.05	0.05	211.52	219.34
2	3	1	9.50	0.03	0.07	0.03	0.07	126.81	292.54
2	3	2	9.50	0.04	0.05	0.04	0.05	185.53	233.34
AVG			9.50	0.04	0.05	0.04	0.05	173.67	225.14
SD			9.50	0.01	0.01	0.01	0.01	40.14	37.99
%CV			9.50	26.63	18.74	26.47	19.58	23.11	16.87
3	1	1	19.00	0.08	0.06	0.08	0.06	353.12	281.66
3	1	2	19.00	0.06	0.08	0.05	0.06	266.48	339.42
3	2	3	19.00	0.08	0.09	0.08	0.08	346.48	389.40
3	2	2	19.00	0.09	0.11	0.08	0.11	378.88	475.95
3	3	1	19.00	0.08	0.08	0.07	0.08	293.51	316.69
3	3	2	19.00	0.07	0.08	0.06	0.08	314.07	363.96
AVG			19.00	0.08	0.08	0.07	0.08	325.42	361.18
SD			19.00	0.01	0.02	0.01	0.02	41.69	67.46
%CV			19.00	12.83	20.53	16.86	19.40	12.81	18.68
4	1	1	38.10	0.14	0.16	0.14	0.16	609.04	690.45
4	1	2	38.10	0.14	0.12	0.13	0.12	590.60	537.42

Level	Replicate	Run	Conc ppm	Amp/ IS1	Meth /IS1	Amp/ IS2	Meth /IS2	Amp/(IS1 /IS2)	Meth/(IS1/ IS2)
4	2	1	38.10	0.15	0.16	0.15	0.16	576.74	612.89
4	2	2	38.10	0.15	0.18	0.14	0.17	638.96	756.47
4	3	1	38.10	0.14	0.18	0.13	0.17	544.95	714.57
4	3	2	38.10	0.17	0.17	0.13	0.14	717.79	729.88
AVG			38.10	0.15	0.16	0.14	0.15	613.01	673.61
SD			38.10	0.01	0.02	0.01	0.02	60.20	82.74
%CV			38.10	9.66	13.19	7.17	13.22	9.82	12.28
5	1	1	95.20	0.40	0.37	0.40	0.37	1764.05	1654.98
5	1	2	95.20	0.45	0.44	0.43	0.42	1986.49	1948.22
Avg			95.20	0.42	0.41	0.41	0.40	1875.27	1801.60
SD			95.20	0.04	0.05	0.02	0.03	157.29	207.35
%CV			95.20	8.39	11.52	5.63	8.76	8.39	11.51
6	1	1	190.50	0.81	0.82	0.87	0.88	2580.02	2610.59
6	1	2	190.50	0.75	0.76	0.82	0.84	2290.37	2328.39
Avg			190.50	0.78	0.79	0.85	0.86	2435.19	2469.49
SD			190.50	0.04	0.04	0.04	0.03	204.82	199.55
%CV			190.50	5.17	4.84	4.15	3.81	8.41	8.08