



ELECTROCHEMICAL AND
ELECTROGENERATED
CHEMILUMINESCENT INVESTIGATION
OF ATSSs

Submitted by

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PhD Thesis

Emre Dokuzparmak

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Signed:

A handwritten signature in black ink, appearing to be 'Emre Dokuzparmak', written over a horizontal line.

Date: 23-06-2021

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Abstract

There is an increasing use recreational drugs in recent years. Indeed, this has become more common place and as such, there is a need for new and portable sensors in forensic field illegal drugs. The need for rapid and reliable detection and monitoring in forensic science has resulted in the development of alternative analytical approaches. Electrochemical sensors are strong candidates for detection sensors and currently electrochemical sensors have a wide range application in forensic applications. In this approach a modified GC electrode with nafion was prepared and voltammetry and electrochemiluminescence (ECL) detection were investigated for the detection of methamphetamine. Methamphetamine (MA) was detected by the modified electrode and the system showed a linear working range of lower concentrations of MA compared with that of the unmodified electrode system. The ECL intensity was found to be concentrations over the range of 5×10^{-8} to 2.5×10^{-4} mol/L. LoD for MA is 1.94×10^{-10} mol/L. This approach was applied in different medium such as saliva and human serum to detect MA and its main metabolites. In addition, this approach was shown to detect two of the main metabolites of MA within a variety of biological matrices. Also, iridium and osmium complexes were used to distinguish MA and its metabolites in samples. However, the modified electrode systems do not show ECL signal against the analytes.

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Publications

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Chapter 1- Introduction and Review of the Literature

1. Introduction

1.1. Drug abuse

Drugs are generally defined as chemical components that have pharmacological or physiological effect on the body.²⁹ The development of new drugs is predominantly for the treatment of diseases. There is an increasing use of drugs as recreational item in recent years and the recreational using of drugs has become very common case. Generally illicit substances are classified within legal systems based upon their effects.²⁹ This project will focus on synthetic drugs known as amphetamine-type stimulants (ATS). ATSS are strong stimulant and hallucinogen and take an important part in forensic field. Some of well-known amphetamine-type stimulants are amphetamine (AMP), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (Ecstasy and known with its chemical name MDMA), 3, 4-methylenedioxyamphetamine (MDA), and 3, 4-methylenedioxyethylamphetamine (MDEA).⁸⁷

The use of recreational drugs has expanded beyond national boundaries. Illicit chemists are synthesizing new derivatives of drugs like different variations of amphetamine-type substances and cannabinoid derivatives. The global estimates of users of amphetamine-type drugs are 0.3 – 1.3 % of the world population ranging 15 to 64 years old. However, the use of illicit drugs in England and Wales is higher than that of lots of European countries and is 8.8 % of the population between 16 to 59 years old.⁴¹ According to a 2017 report published by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), England, Scotland and Ireland are major stopping-off points for gangs seeking to supply the

European drugs market. Criminal groups have created routes for the sale of methamphetamines (Figure 1.1).

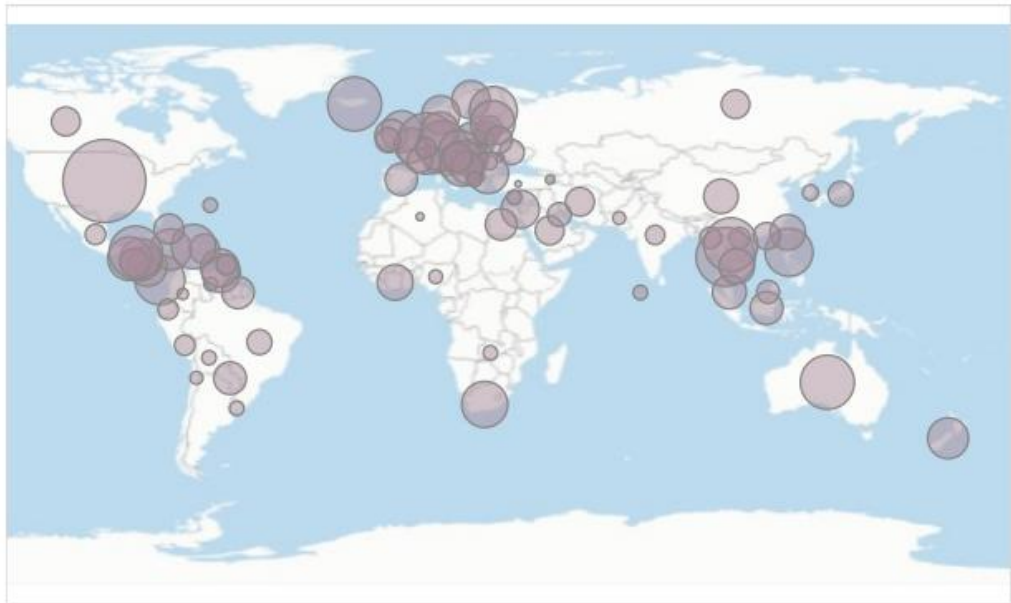


Figure 1.1. Illustration of the number of individual new psychoactive substances (ATs) reported to the World Drug Report 2020. Balloons symbolise the amount of ATs in that location.⁹²

ATs are one of the most common illicit drug classes. According to the 2020 World Drug Report by The United Nations, the ATs has an increasing illegal usage most commonly worldwide last years.⁹² Also, this report emphasises that methamphetamine, in the period 2014–2018, creates 71 per cent of the total quantity of ATs seized globally, which means that the amount of methamphetamine produced in illegal laboratories increased by approximately 300 % in a narrow period. In addition, there is a significant degree of intraregional methamphetamine trafficking occurring in North America, Western and Central Europe and East and South-East Asia.³⁰ The European “ecstasy” market seems to be expanding and the market for

“ecstasy” in Europe is estimated to be worth at least 0.67 billion euros per annum. European “ecstasy” seizures increased from 2013 to 2015, reaching almost 4 tons, while recent surveys indicate an overall increase in the use of “ecstasy” in Europe.³⁰ Methamphetamine has the second highest prevalence of drug abuse after cannabis, with estimates of 35 million users worldwide.¹

In the UK, two main legislations have been used to control on drug abuse and they are the Misuse of Drugs Act (1971) and the Misuse of Drugs Regulations (2001).⁴²⁻⁴³ The Misuse of Drugs Regulations (2001) shows the conditions about how controlled substances can be used for medical and research applications.⁴² The Misuse of Drugs Act (1971) includes the name of substances which are potential for abuse and limitations of use of these substances. These substances are divided into three different drug classes (A, B and C). This legislation also includes controlled substances’ stereoisomers, esters, salts.⁴³ Methamphetamine falls into ‘Class A’ controlled substances within the Misuse of Drugs Act (1971). This class substance has a restricted use as therapeutic drug and high potential for abuse.

New types of drugs are developed by illegal labs and forensic scientists encounter new variation of illicit drugs and sometimes completely new metabolites of drugs. For the control of new type and variations of drugs, the legislations must be kept up to date. Also, the detection of new types and variations of drugs is generally hard to identify because of more complex structure or different metabolites of new drugs. In this purpose, reliable and rapid analytical techniques are required for identification and characterisation of new drugs.⁶⁰

1.2. Amphetamine-type stimulants

Amphetamine (alpha- methylphenethylamine) is a chemical structure that has a phenethylamine (PEA), as a backbone, and a methyl on α position of the backbone (Figure 1.2). Amphetamine has two enantiomers (racemic mixture 1:1) (L-amphetamine and D-amphetamine). They are known as levoamphetamine and dextroamphetamine and generally are in the form of solid salts.

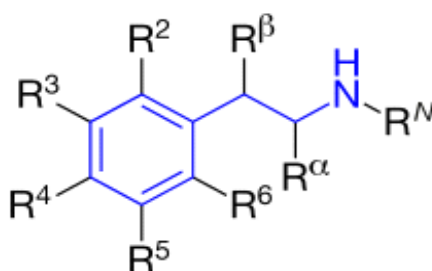


Figure 1.2. *The backbone of phenethylamine (PEA) and all active positions on it*

Most known amphetamine type substances fall into stimulants, empathogens and hallucinogens. Some of them are central nerves stimulants and are used for treatment of some illnesses like obesity and hyperactivity at therapeutic doses and they may be used as a drug at higher doses than therapeutic doses, as well as doping agents in sports. Some the popular analogous of illicit amphetamines-like drugs are 3,4 methylenedioxymethamphetamine, its known name is “ecstasy”, methylenedioxyamphetamine, methamphetamine (ice), methylenedioxyethyle-amphetamine (MDEA). The molecular structures of these common ATS are shown in Figure 1.3.

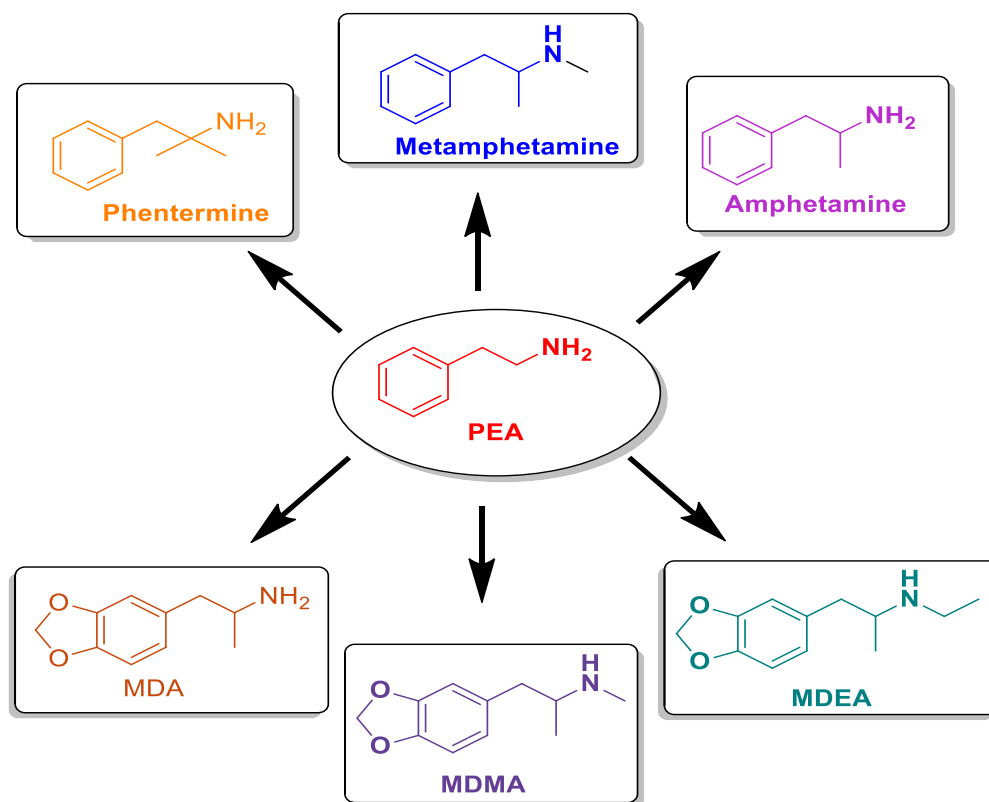


Figure 1.3. Molecular structures of amphetamine, methamphetamine, 3,4-methylenedioxy-amphetamine (MDA), methylenedioxyethyleamphetamine (MDEA) and 3,4-methylenedioxy-methamphetamine (MDMA).

Dextroamphetamine is 'right-handed' enantiomer of the amphetamine molecule. Dextroamphetamine is a potent central nervous system (CNS) stimulant and amphetamine enantiomer that is prescribed for the treatment of attention deficit hyperactivity disorder (ADHD) and narcolepsy.⁸⁰ It is also used as an athletic performance and cognitive enhancer, and recreationally as an aphrodisiac and euphoriant.⁴⁶

Levoamphetamine, also known as levamfetamine (INN), (R)-amphetamine, (–)-amphetamine, and L-amphetamine, is a central nervous system (CNS) stimulant known to increase wakefulness and concentration

in association with decreased appetite and fatigue. Pharmaceuticals that contain levoamphetamine are currently indicated and prescribed for the treatment of attention deficit hyperactivity disorder (ADHD), obesity, and narcolepsy.

ATS are psychostimulants and generally have different effects on central nervous system (CNS) depending on the enantiomeric form of the substance.⁷⁴ They can lead to increased synaptic levels of neurotransmitters in the brain such as dopamine and serotonin.¹² When ATS are taken, dopamine levels in the brain rise quickly and this increase causes a feeling of 'euphoria'. This feeling is the main reason for recreational abuse of ATS. ATS also show some side-effects such as high blood pressure, heart rate and temperature. Therefore, long-term abuse of ATS can cause cardiovascular problems.¹²

Determination of amphetamines is important within medicine fields due to the toxicological and addictive effects of amphetamines. In this respect, there are lots of methods used effectively in these fields. Chromatographic techniques utilized include gas chromatography- mass spectrometry (GC-MS), high-performance liquid chromatography with mass spectrometry or tandem MS (HPLC-MS or HPLC-MS/MS, respectively), high-performance thin-layer chromatography (HPTLC) and capillary electrophoresis (CE). Immunoassays are used very effectively to determinate the presence of amphetamines in urine, blood, and alternative matrix like hair, sweat and saliva in therapeutic and illicit drugs.³⁵ To illustrate, sport anti-doping analysis are famous ones in forensic science.

1.2.1. Methamphetamine

Methamphetamine is a potent central nervous system stimulant and is mainly used for recreational drug. There are two enantiomers of methamphetamine, and they are levo-methamphetamine and dextro-methamphetamine. In pure amine forms, these enantiomers are equal in the mix and show racemic property. Dextro-methamphetamine is a much stronger central nervous system than levo-methamphetamine.²¹ Dextro-methamphetamine is generally in the form of a hydrochloride salt and this salt is called as 'ice' and 'crystal math'. The crystal math allows users to inhale the vapour of methamphetamine. This ability of hydrochloric form of methamphetamine enables higher and faster recreational effect.²¹

Methamphetamine has the ability of substitute for monoamines at membrane-bound transporters due to its structural similarity. This substitutional impact effects the mechanism of the re-distribution of monoamines and leads to release of dopamine and serotonin in the synapses.²¹ The users rapidly have euphoria as desired by abusers of the drug.⁸²

The chemical and pharmacological characteristics of methamphetamine have led to become the second most popular illicit drug worldwide.⁵⁶ Due to its worldwide use, there is a requirement for efficient analytical techniques that enable the direct detection of methamphetamine within complex sample matrices to enable high-throughput screening.

The primary metabolites of MA are amphetamine (AMP) and *p*-hydroxymethamphetamine. They are formed by *N*-dealkylation and aromatic hydroxylation, respectively (Figure 1.4).

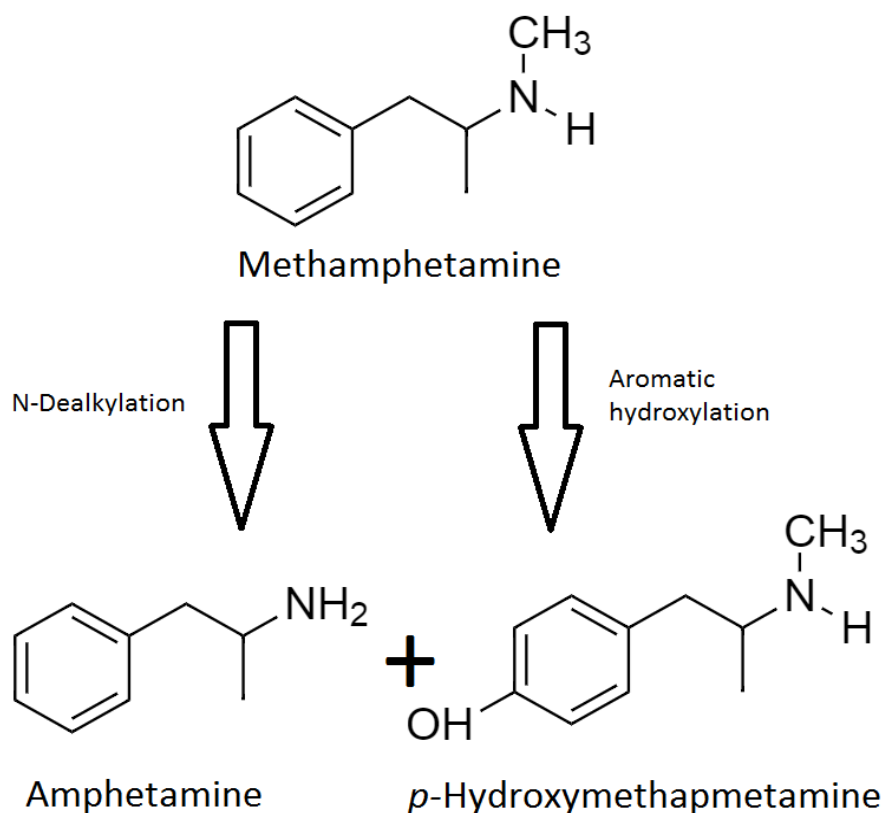


Figure 1.4. Metabolism of methamphetamine

1.3. Introduction to Electrochemistry

The majority of electrochemical detection in forensic electrochemistry is grouped in two different methods. Potentiometric methods and potentiostatic methods involving the use of a potentiostat and measuring a change in voltage by oxidation or reduction reactions in a electrolytic cell.⁴⁷ An electrochemical cell consists of a standard 3 electrode set-up including a working electrode, a counter electrode and a reference electrode (Figure 1.5).

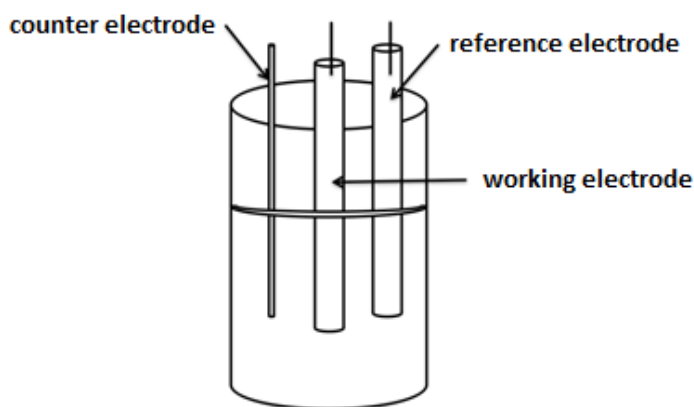


Figure 1.5. Typical electrochemical cell, WE, working electrode; CE, counter electrode; and RE, reference electrode.

The working electrode measures oxidation and reduction reactions occurring at the electrode surface. The counter electrode is used to allow current to flow by providing electrons for reaction of oppositely charged species. A reference electrode provides a well-defined redox couple with a fixed potential. Occurring potential of oxidation and reduction at working electrode is compared to the potential at the reference electrode.⁴⁷

1.3.1. Cyclic voltammetry

Cyclic voltammetry (CV) is a very popular technique because cyclic voltammograms show certain well-defined characteristics for an electrochemical reaction typical of the redox reductions occurring and CV enables multiple detections in one measurement (qualitative), and estimation of their concentration in solution (quantitative). CV also involves monitoring the current response of a small stationary electrode in an unstirred solution which is excited by a triangular potential wave form (Figure 1.6).

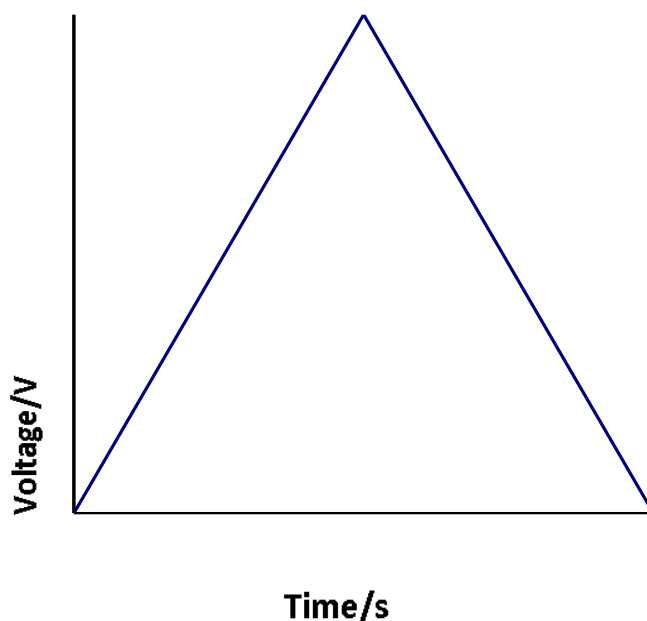


Figure 1.6. *Cyclic voltammetric excitation signal used to obtain voltammogram.*

The intensity of a redox peak will be dependent upon concentration and so can be used to determine the concentration of an electrochemical active substance.⁶⁸ In CV, a fixed potential range is employed, and the voltage is scanned from a lower limit to an upper limit and the current (I) is recorded as a function of the potential (E). Then, the scan is reversed, and the voltage is swept from the upper limit to the lower limit. As the potential is swept back and forth, current flows through the electrode that either oxidizes/reduces the analyte and the current between the working electrode and the counter electrode is measured and plotted against the potential. A peak in the I vs. E output denotes a flow of electrons between the analyte and the electrode associated with a change in the oxidation state of the electroactive analyte. A cyclic voltammogram is produced upon plotting both the anodic and cathodic scans on the graph as shown in Figure 1.7 and 1.8.⁶⁸

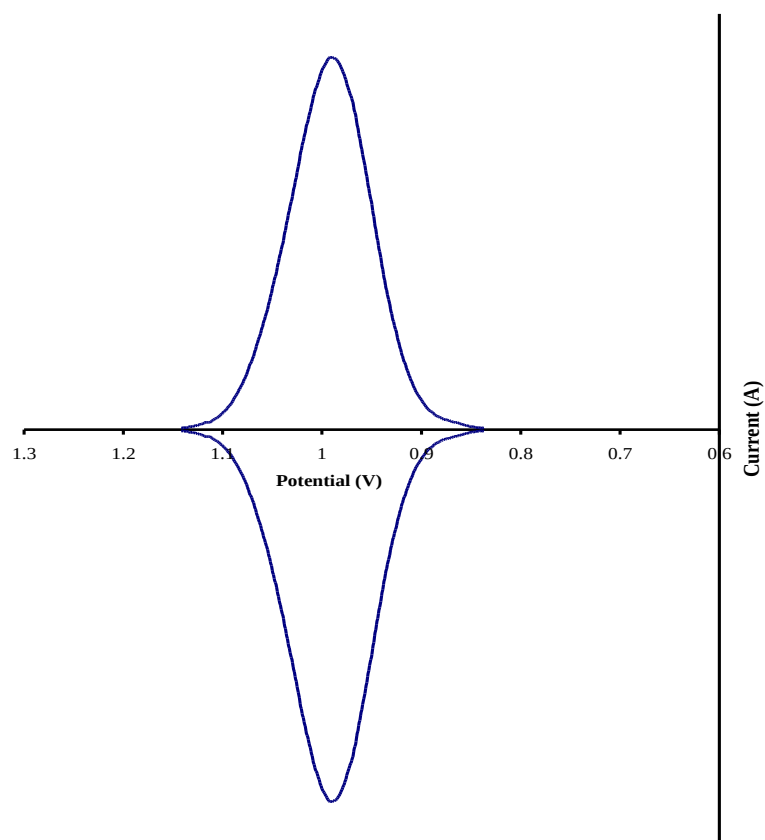


Figure 1.7. Background corrected cyclic voltammogram showing theoretical response for an electrochemically reversible couple that is confined on the electrode surface.⁶⁸

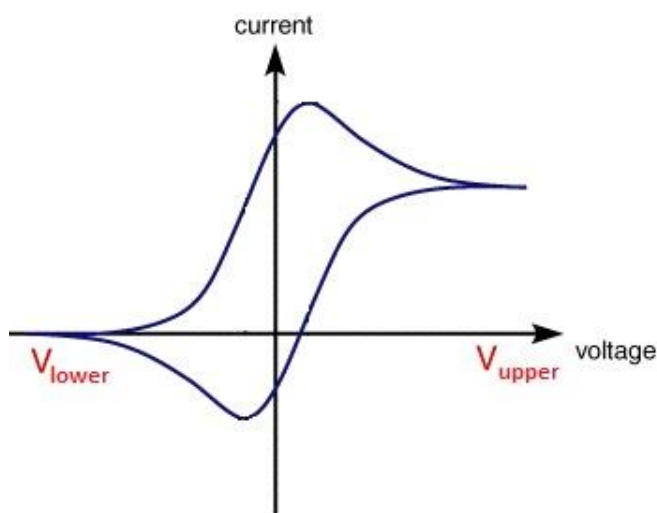


Figure 1.8. A typical voltammogram of current versus voltage, V_{lower} is the lower limit of voltage and V_{upper} is the upper limit of voltage

When a potential is applied to a surface modified electrode the current rises from essentially zero to a peak value and then back to zero. For an ideal system no peak to peak separation is expected. Under the conditions of finite diffusion in which the redox composition of the layer is in thermodynamic equilibrium with the electrode potential, (i.e. the Nernst condition), it will be observed for all electrochemical reversible reactions at sufficiently slow scan rates such that all electroactive centres undergo redox transformations on the time-scale considered.

The model for ideal reversible voltammetric behaviour under such conditions has the following features;

$$i_p = \frac{n^2 F^2 A \Gamma \nu}{4RT} \quad (1.1)$$

$$E_{p,c} = E_{p,a} \quad (1.2)$$

$$FWHM = \frac{90.6}{n} mV \quad (1.3)$$

$$\frac{i_{p,c}}{i_{p,a}} = 1 \quad (1.4)$$

Where FWHM is the full width at half maximum, n is the number of electrons passed, F is Faraday constant, Γ the total electroactive coverage, A is the electrode area, v is the scan rate, R the gas constant and T is temperature. These are shown in Figure 1.9.

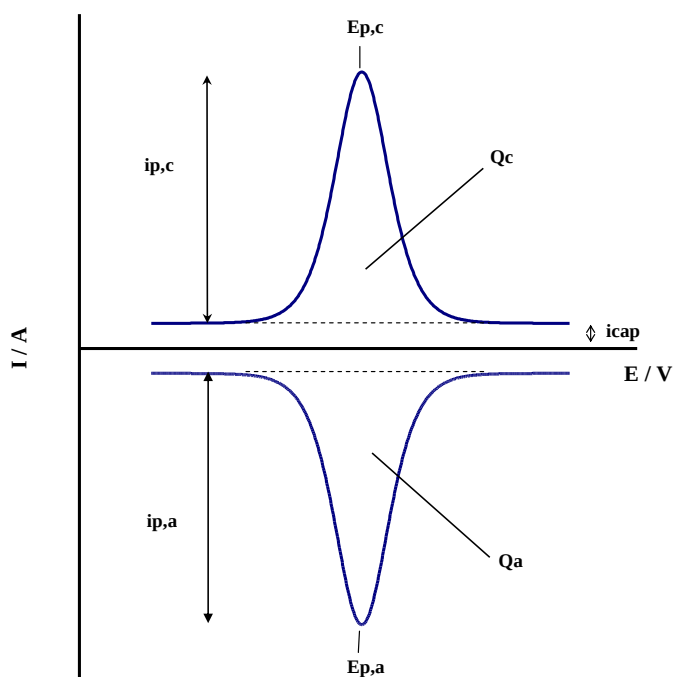


Figure 1.9. Cyclic voltammogram of a chemically modified electrode with a monolayer of an electrochemically reversible mediator.

Under these conditions of exhaustive oxidation/reduction of the modifying layer the Faradaic charge, Q , under the current potential curve gives the quantity Γ (mol cm^{-2}) according to the expression;

$$\Gamma = \frac{Q}{nFA} \quad (1.5)$$

For a polymer or thicker film under these conditions of finite diffusion, a plot of scan rate versus peak current will be linear. At higher scan rates which provide time only for partial electrolysis of the total number of redox sites during each linear scan, a transition to semi-infinite diffusion control occurs and a $v^{1/2}$ dependence on peak current is observed. Under these semi-infinite conditions the following behaviour is observed;

$$\Delta E_p = \frac{57}{n} mV \quad (1.6)$$

In most films, electrochemical charge transport is thought to occur by electron self-exchange reactions between neighbouring oxidised and reduced sites. This electron hopping process is mathematically representable by diffusion laws in which the charge transport diffusion coefficient, D_{ct} , is introduced as a measure of its rate.

The efficiency and performance of the overall CV procedure is largely based upon the selection of the working electrode, pH and the solvent. The type of working electrode used is based upon the redox behaviour of the analyte and the potential range required.⁸³

A limitation in the use of CV is that the voltammograms obtained can be difficult to interpret. Furthermore, the identification of anodic and cathodic peaks can be difficult at low analyte concentrations.⁸³

1.4. Electrochemiluminescence

Electrogenerated chemiluminescence (ECL) is the production of light from electrochemically produced species form via a high-energy electron transfer reaction.³¹ ECL emission occurs in the visible part of spectrum as a consequence of fast and highly exo-energetic electron transfer reactions between a strong electron donor and electron acceptor which in turn results in the generation of excited states.⁸⁹

ECL is a form of luminescence. However, luminescence in CL is initiated by the addition of one necessary reagent to the other. Luminescence in ECL is initiated and controlled by altering an electrode potential (Figure 1.10).

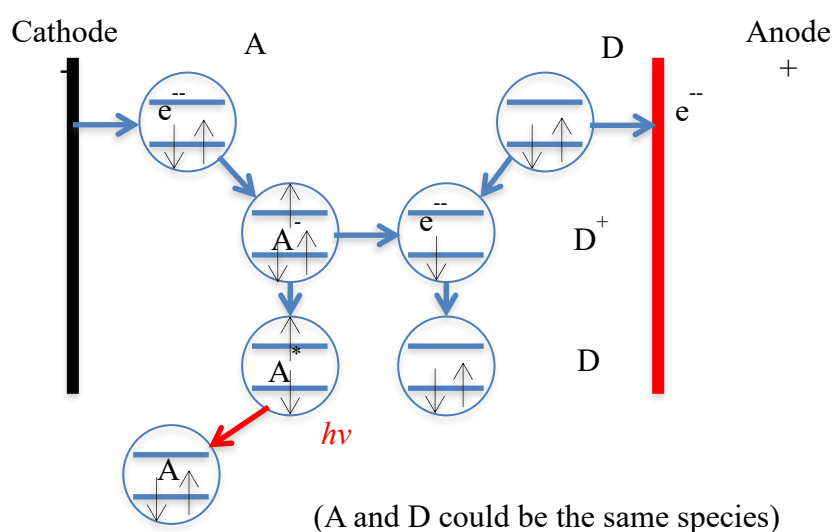


Figure 1.10. ECL process. Adapted from Miao (2008).⁵⁷

ECL has a number of important advantages over more conventional chemiluminescence (CL) techniques. Firstly, no external light source is required and therefore instrumentation is simple, background light intensity

is minimal and scattered light and interferences from emission by impurities is minimised. Secondly, certain reactants can be regenerated at the electrode, allowing them to undergo ECL reactions more than once thus greatly improving the sensitivity of the technique. This allows the detection of analytes at very low concentrations (picomolar).⁵⁷

One of the major advantages of ECL is the greater control over both the position and time of ECL reactions. ECL emission occurs only in the immediate vicinity of the electrode, which is beneficial for selectivity, multi-analyte detection using multiple electrodes, and sensitivity. Control over the timings of the reaction improves reproducibility.⁵⁷

These advantages have resulted in the application of ECL across many diverse research fields, including food and water testing, clinical diagnostics and bio-warfare agent detection.^{69, 79}

1.4.1. Ion annihilation ECL

Ion annihilation ECL involves the generation of the oxidized and reduced forms of a radical ion through the application of a potential. They can be formed sequentially at the same electrode using potential sweep/step methods. Initially, the electrode potential is stepped to produce a radical anion, which remains in the diffusion layer surrounding the electrode. The potential is then altered so as to produce the radical cation, which diffuses away from the electrode. At this secondary potential, the radical anion is oxidized and thus its concentration at the electrode diminishes. This results in back diffusion of the radical anion in the bulk to the electrode, causing the interaction and reaction with the electrogenerated radical cation (equations 1.7 to 1.12).⁵⁷



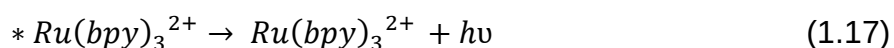
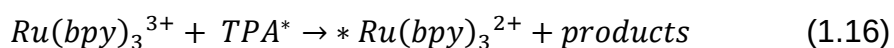
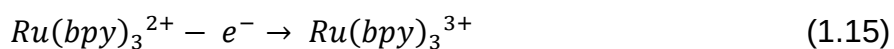
The major advantage of the annihilation pathway is that only the ECL species, solvent and electrolyte are required to generate light and the system is therefore simple and cost-effective.

However, this system has some limitations, namely, for emissions in the visible region (~400-700 nm) and therefore, for annihilation ECL to occur, the potential window of the system must be large enough to allow the generation of stable radical anions and cations.⁵⁷ Additionally, quenching of excited states by dissolved O₂ can significantly affect the annihilation ECL response and this must be removed from the system prior to analysis.⁹¹

1.4.2. Co-reactant ECL

Co-reaction ECL involves the addition of a reagent, often referred to as the co-reactant, to the system. A potential is applied at an electrode causing the oxidation or reduction of both the luminophore and the co-reactant to form radicals. The electrogenerated co-reactant species then immediately decomposes to form a powerful reducing or oxidizing

intermediate.⁵⁷ This is because electrochemical oxidation involves the loss of an electron and as such an electron ‘hole’ exists in the highest occupied molecular orbital (HOMO) of the oxidized species, creating a strong oxidising agent. Likewise, the addition of an electron into the lowest unoccupied molecular orbital (LUMO) of the reduced species increases the reducing power of that species. These strongly reducing or oxidizing intermediates can then react with the oxidized or reduced luminophore to produce an excited state. After the light emission has occurred, the luminophore can be regenerated, however, the co-reactant is consumed during the electrochemical reactions (equations 1.13 to 1.17).



[Ru(bpy)₃]²⁺/tripropylamine (TPA) system, the most common ECL system, is currently the basis for the commercial ECL immunoassays and DNA analyses. The ECL intensity of the Ru(bpy)₃²⁺/TPA system strongly depends on the solution pH. A large number of ECL applications in biological systems appear in literature, owing to its feature that the maximum value of the ECL intensity occurs at around pH 7.5, which is exceptionally suitable for applications in biological systems.²⁸ To be a good ECL co-reactant, a number of criteria need to be met, which include

solubility, stability, electrochemical properties, kinetics, quenching effect, ECL background, etc. The most important factor is the electrochemical properties of the co-reactant. The co-reactant should be easily oxidized or reduced with the luminophore species at or near the electrode and undergo a rapid chemical reaction to form an intermediate that has sufficient reducing or oxidizing energy to react with the oxidized or reduced luminophore to form the excited state.⁵⁷

1.4.3. Detection and Quantitation Limits (LoD and LoQ)

The quality of an analytical method is depended on purpose, sensitivity, analyte stability, simplicity of the analysis, time and cost-effective structure. The quality of analytical data is a key factor in the success of an analytical method development. In this context, validation procedures are vital in development of analytical method and detection of drugs and pharmaceuticals. Validation process are indispensable for reliable monitoring of integrity, strength, quality and potency of the products. Limit of detection (LoD) and limit of quantification (LoQ) are two important performance characteristics in method validation. LoD and LoQ parameters are related but have distinct definitions and should not be confused.

There are some terms that are used to define LoD and LoQ but simply the LoD is known as the lowest concentration of an analyte that can be detected in a sample but there is no necessity for quantification of the analyte under the test conditions. The LoQ is the lowest concentration of an analyte in a sample that can be detected with acceptable precision and accuracy under the test condutions.⁷¹

Also, an assay or an analytical method has a dynamic range that extends from some lower limit to some upper limit. Typically, an assay not capable of accurately measuring analyte concentrations down to zero. That is due to the fact that sufficient analyte concentration must show an analytical signal that can be distinguished from analytical noise which is the signal observed in the absence of the analyte.⁷⁸

However, there are some common estimation methods for LoD and LoQ and they are;

- Visual definition
- Calculation from the signal to noise ratio
- Calculation from the standard deviation of the blank
- Calculation from the calibration line at low concentrations

$$\frac{DL}{QL} = \frac{F \times SD}{b} \quad (1.18)$$

Where

DL: Detection limit

QL: quantification limit

F: Factor of 3.3 and 10 for *DL* and *QL*, respectively

SD: Standard deviation

b: slope of the regression line

The estimated limits should be verified by analysing a suitable number of samples which contain the corresponding concentrations of the analyte. Also, relevant chromatograms should be reported. In this study, Visual definition method was used to estimate LODs of the present study.

1.5. Traditional Detection Methods

There is a wide range of applications within the scope of forensic science. By using comprehensive chemical and analytical techniques in forensic labs forensic science specialists assess the evidence. In this context, scientists need up-to-date and reliable analytical techniques to identify and detect the samples swiftly and with great accuracy.⁷⁵ The need for new and more effective systems in forensic field becomes more of an issue because unknown derivatives of illegal drugs, which have more complex structure and are more hazardous for human health, have come to exist over time. Scientists are looking for advanced approaches to solve the current problems.⁷⁵

In the phase of choosing an appropriate method and technique, there are lots of factors which are key to assess which analytical method or technique is more suitable. Sensitivity, selectivity, repeatability and cost are some of the important factors to determine performance of an analytical technique.¹³

One of the most common problems in clinical and forensic analysis is interferences in complex sample matrix like whole blood and urine. These interferences make the analyte harder to analyse due to the fact that the interferences in the matrix interact with the system and give false-positive response. Generally, urine samples are preferred for analytical practices

because urine samples have relatively less interferences in the matrix. However, there is another problem in urine samples, and it is because target analytes in urine sample are in metabolised form.⁵⁵

Seized 'street' samples generally contain different type of ATS and some agents that have interference effect in analyses. The characterisation of potential interferences is very important for reliable response from the analytical method.⁵⁵ Furthermore, it may be a requirement to identify the stereoisomeric composition of the ATS.

1.5.1. Screening methods

Detection of illicit drugs are generally costly and there is a tendency to use cost effective analysis for detection of drugs. 'Street' samples are analysed in forensic laboratories by screening methods to determine the presence of a drug class and decide a suitable analytical method for further analysis.⁶⁰

Presumptive drug tests can sometimes be used for ATS because they are fast and cost-effective techniques. Presumptive tests are generally portative, and they can be used easily in crime scene. In these tests, the presence of the ATS can be determined by using a colour change in the present of specific reagents.⁶⁰ Due to non-specific nature of these tests, positive and negative controls must be included to ensure the system is working correctly. Despite presumptive tests are used in crime scene due to portability of them, there are limitations for use of presumptive tests to recognize drugs. To illustrate, in the presence of drugs in a sample, a colour change is observed. However, presumptive tests cannot distinguish between MDA and MDMA. Furthermore, presumptive tests suffer from

poor sensitivity and can show a colour change in the presence of adulterants, cutting agents and additional drug molecules.¹⁹ In the present study, it is seen that ECL suffer from relatively low selectivity and the present system can not distinguish MA and its main metabolites in aquatic medium. Therefore, ECL methods which suffer from selectivity may fall into screening methods. When ECL method is very selective and sensitive, then that ECL method can be under confirmatory methods. The key factor to categorise any ECL system is selectivity.

1.5.2. Confirmatory methods

As it was mentioned previous section, the screening methods cannot identify qualitative and quantitative properties of a drug. Also, the samples in forensic labs generally have complex matrix. There is a need to confirmatory analysis for detection of the analyte selectively and characterisation of all components.⁴⁸ For the identification of an unknown sample, it is a UK legislative requirement that identification must be achieved by two physio-chemically different analytical.⁹

Gas chromatography, high-performance liquid chromatography and mass spectrometry (MS) are commonly used for forensic cases. These techniques are generally combined with another technique (one chromatographic and one spectroscopic) such as gas chromatography and mass spectrometry (GC-MS) or high-performance liquid chromatography and mass spectrometry (HPLC-MS).³³ In addition, their ability to analyse multiple analytes in a single analytical run enables higher sample throughput and more efficient use of laboratory instrumentation.⁵⁵

1.5.2.1. Gas Chromatography

Gas Chromatography is a common type of chromatography used in analytical chemistry for separating and analysing compounds that can be vaporized without decomposition.

An optimised method was validated for some ATS, with satisfactory linearity and selectivity ranging between 50 and 2000 ng mL⁻¹, and limits of detection (LOD) and low limits of quantification (LOQ) ranging between (5–25) and (25–100) ng mL⁻¹, respectively.² In another study, small volume liquid extracts of some ATS were prepared from saliva. LODs were less than 5 ng/mL in saliva using GC/MS and LOQs were 10 ng/mL.¹⁴ The analysis of real blood and urine samples examined in 22 forensic toxicological cases showed a range of LOD (between 0.09 and 0.81 ng/mL) and LOQ (between 0.26 and 2.4 ng/mL) and high selectivity for ATS.⁹⁴

Although GC has a wide usage in forensic field, there is a disadvantage of GC in the analysis of ATS. There is the requirement of derivatisation because ATS show poor resolution and peak tailing.⁴⁸

ATS with low thermal stabilities can lead to degradation products which may cause interference or the detection of an analyte concentration that is lower than present.⁴⁴ The thermal stability and chromatographic behaviour of the ATS can be improved by the addition of a derivatisation step leading to a molecular weight increase and derivatisation of the polar hydroxide and amino functional groups respectively. The most commonly utilised derivatisation reagent in the analysis of the ATS is heptafluorobutyric anhydride (HFBA).⁴⁴

1.5.2.2. Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that ionizes chemicals and sorts their ion forms based on their mass to charge ratio (m/z) which can result in high sensitivity selectivity in the detection of unknown samples. The most commonly utilised detector in confirmatory analysis has been mass spectrometry (MS) as a result of its wide dynamic range, increased sensitivity and high degree of selectivity in multiple analyte analysis. Mass spectrometry generally is combined with gas chromatography (GC) or high-performance liquid chromatography (HPLC).

Gas chromatography - mass spectroscopy (GC-MS) is the most frequently used analytical technique for identification of qualitative and quantitative properties of organic substances in complex matrices. GC-MS is essential in the field of forensics, environmental science and medical research.⁷³

GC-MS is one of the most analytical powerful confirmatory technique in forensic science and is called as 'gold standard'.⁶⁵ GC-MS is capable of multiple degrees of separation and high selectivity and can identify analytes in a complex matrix.¹⁷

Despite GC-MS has advantages for forensic science, there are some drawbacks such as the requirement of derivatisation of ATS and difficult of movement of the ATS analyte ions out of a region of low m/z values.²⁴

1.5.2.3. High performance liquid chromatography

High performance liquid chromatography (HPLC) is very applicable alternative tool to GC-MS because HPLC can detect qualification of polar, non-volatile and high molecular mass compounds. Also, this technique

does not need derivatisation process.⁵⁹ HPLC allows the direct analysis of aqueous samples. Thus, HPLC becomes a cost-effective technique. HPLC uses similar principle to that of GC but in HPLC the sample move through the column by using a solvent known as the mobile phase.¹⁹

In the literature, there are lots of studies about ATS. To illustrate, in a study for development and validation of two LC-MS/MS methods for the detection and quantification of amphetamines, the limits of detection and quantification for all drugs were less than 10 ng/mL and less than 25 ng/mL. Calibration curves ranged from 25 to 200 ng/mL for amphetamines, designer amphetamines, and benzoylecgonine, from 25 to 250 ng/mL for benzodiazepines, from 12.5 to 100 ng/mL for morphine, codeine, and dihydrocodeine, and from 5 to 50 ng/mL for buprenorphine and norbuprenorphine.⁶⁶ In another study, a liquid chromatography–tandem mass spectrometry method has been developed and validated for confirmation and quantification of three amphetamine-type stimulants (ATS) in oral fluid (OF), namely fenproporex (FEN), diethylpropione (DIE) and methylphenidate (MPH). Calibration curves were linear throughout the concentration range from 2.5 to 90 ng mL⁻¹. the limits of detection were 2.5 and 0.5 ng mL⁻¹, respectively, for all ATS.²²

The major limitation associated with the co-elution of the ATS using HPLC-MS is that extensive method development of the chromatographic parameters is required to achieve resolution of neighbouring ATS peaks. Furthermore, these methods can require long analytical run times in the region of ~20 minutes which can have a detrimental effect on sample throughput.⁵⁹

It is obvious that the screening methods and the confirmatory methods for the analysis of the ATS have some limitations. The screening methods have problems about selectivity and sensitivity. On the other hand, the confirmatory methods have drawbacks of cost and time. Electrochemical methods may be an alternative screening approach to identify qualitative and provide semi-quantitative analysis of the ATS within complex matrices at the crime scene and without the need to complex sample preparation.

1.6. Alternative Detection Methods

The chemical sensors are basically analysers that give an analytical response (electrical signal) to a particular analyte selectively. Concentration of a specific chemical in a medium can be detected by a biomaterial or a chemical component. Sometimes a combination of them are used in a sensor system.⁹⁵ The international Union of Pure and Applied Chemistry (IUPAC) Commission reported that biosensor is a subgroup of chemical sensors.³⁹ Biosensors are systems with a bioactive part and have ability to convert the interaction between the bioactive component and analyte to a meaningful signal. Biosensors arouses attention due to their simple and cost-effective characteristic. Biosensor applications are a wide range of analyte scale from DNA, protein to ions, drugs, and toxins.⁵¹

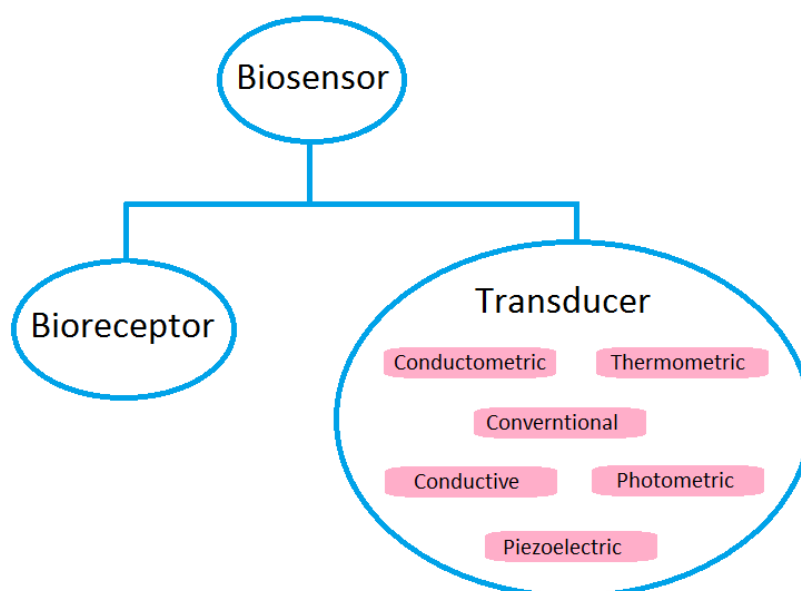


Figure 1.11. Different types of transducers for biosensors

Essential biosensor components are a biological recognition element, a transducer, an electrical amplifier, and data handling system. Biological material in a biosensor may have interest in a group analyte or a specific affinity for an analyte. Biosensors are generally classified by their type of transducers including electrochemical, optical, thermal, mass-sensitive types and in theory a proper biological component is used as an interface compound to transducer in order to measure the analyte.⁷⁰ Despite there are lots of sub-class biosensor, main types of biosensors are electrochemical, amperometric, potentiometric, conductometric, thermometric and optical biosensors, etc. (Figure 1.11).

1.6.1. Immunoassays / Aptamers

Aptamers are in the form of DNA, RNA, proteins or peptides which have high affinity for a target analyte in a matrix. Aptamers can be programmed for viruses, whole-cell target or drugs.^{37,96} As a simple explanation of aptasensor, an aptamer is immobilized on the surface of an electrode as an active sensing element. As a result of the interaction between aptamer and analyte, changes in signal are measured, and target analyte is determined similar to other biosensors.

In a lot of ways, aptamers imitate antibodies. Due to their high binding affinity for target molecules and specificity, aptamers function in the same way as antibodies for sensor.⁹⁶ Aptamers are chemicals, and this property makes them more advantageous than antibodies. Firstly, aptamers are chemically synthesized. Synthesis of aptamers is economical and highly reproducible, compared to the production of antibodies in animals and cultured cells.⁹⁶ Second, specific modifications in antibodies are difficult. Whereas, aptamers are easily modifiable at different function groups.

Third, antibodies must be carefully used in biosensor applications to avoid irreversible denaturation or loss of activity because antibodies are not generally endurable to harsh conditions like the presence of acid or base, organic solvent (alcohol, chloroform etc.). In addition, the conformational flexibility of aptamers makes them reusable, and they can be restored to the original structures and after denaturation.⁹⁶

Peptide aptamers are artificial proteins that can bind specific target molecules. These are isolated by using combinatorial libraries and generally improved by directed mutation or selection. *In vivo*, peptide aptamers bind targets and show biological activity towards the desired target.

A combinatorial library is used to identify biologically active peptides successfully. This combinatorial approach contains three methods, and these methods are briefly that; peptide libraries are synthesized and cleaved from the solid support to be screened as free compounds. Synthetic combinatorial libraries of peptides are assayed on their solid support. A phage display, which enables the selection of clones of interest rather than screening, is used to make the standard protocol easier.⁹⁶

Peptide aptamers show high specificity and low risks of systematic toxicity. The advantages of peptomers are high affinity, specificity, minimized drug-drug interaction, relatively low cost, easy synthesis and suitability for long storage times.⁸ To illustrate, a label-free peptide aptamer-based biosensor for TNT was reported and the peptomer was immobilized on a gold electrode by using Au-S interaction. On incubation with different concentration of TNT, the peptomer on the electrode bound

to TNT and folded, which hindered the electron transfer of the redox probe, $\text{Fe}(\text{CN})_6^{3-/4-}$, leading to an increase in the electron transfer resistance. the change in electron transfer resistance and the logarithm of the TNT concentration from 0.44 to 18.92 pM, with a detection limit of 0.15 pM, was obtained.⁹⁶

Nucleic acid aptamers are a unique class of oligonucleotides because they are different from other oligonucleotides and these structures have high affinity for specific targets. They are synthetic RNA or DNA which have well-defined nanostructures. Unlike antibodies, nucleic acid aptamers are more durable against harsh conditions. This characteristic makes nucleic acid aptamers very useful biomaterials for unique goals in biosensor applications.³⁵ To identify nucleic acid aptamers, SELEX protocol is used. Nucleic acid aptamers have some advantages and are synthesized within hours with highly purity and display great thermal stability affording long self-lives without loss of activity, easy transport and storage and ability to return to a native conformation.³⁵ In many types of biosensing platforms, nucleic acid aptamers have been employed as affinity receptors in combination with various signal transduction strategies including colorimetry, chemiluminometry, electrochemistry, fluorometry, and fluorescence anisotropy.³⁵ To illustrate, An ECL-AB biosensor for ATP detection with high sensitivity and specificity was developed. The ds-DNA was labelled with a ruthenium complex at the 3 terminus of cDNA, followed by the immobilization of this ds-DNA onto Au electrode surface through the 5 -HS on the cDNA. In the presence of ATP, due to the ATP binding to the aptamer, the aptamer molecules dissociated from the ds-DNA complex, which resulted in the formation of stem-loop structure of the single-stranded

cDNA and led to the increase of the ECL signal. Also, the ECL-AB biosensor was regenerated by immersing the Au electrode into a different solution for 60 min. the regenerated electrode became available for another assay.⁸⁸

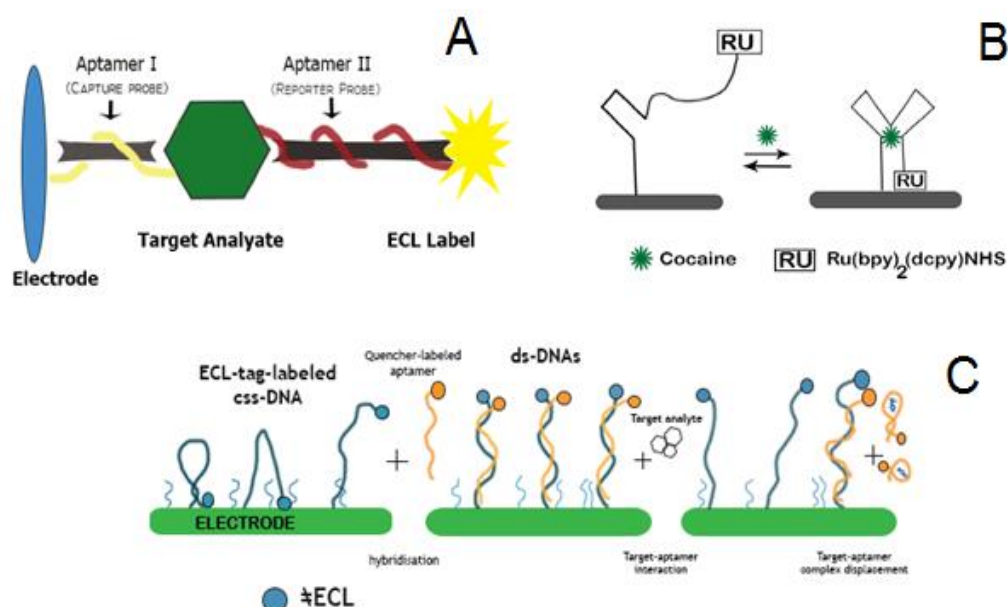


Figure 1.12. Diagrammatic demonstration of **(A)** sandwich type aptasensor (aptamer 1 is capture probe, aptamer 2 is reporter probe modified with ECL label), **(B)** structure-switching of ECL-labelled aptamer, **(C)** displacement of complementary aptamer

ECL aptamer -based sensors (ECL-AB biosensors) are generally categorised by binding sites that have affinity for target molecule. For the detection of target molecules which have more than a binding site, sandwich-type approach is usually used.³⁵ Single-site assays can also be used for detection of a target molecule with a single binding site. This group involves two subgroups (labelled and label-free approaches). A label is defined as any foreign molecule that is chemically or temporarily attached

to the molecule of interest to detect molecular presence or activity. Fluorescent, chemiluminescent, and nanoparticle labelling usually involves covalent bonding through coupling chemistries, while some of electrochemically active probe labelling requires only temporary attachment of intermolecular bonding.³⁵ Label-free detection methods utilize molecular biophysical properties such as molecular weight and molecular charge to monitor molecular presence or activity. Label-free approach is practicable for target molecules with low affinity and more direct information can be acquired, as the methods use only native structures and ligands.^{35,75,88} Despite the fact that CL aptasensors have a respectable success in clinical diagnostics, this approach has not been exploited in forensic research. This is due to the limited number of aptamer sequences, the need for high investment and limited studies for the usage of aptasensors in complex matrix. New developments in aptamer selection and sequencing have encouraged scientists to design aptaseonsors for forensic applications.⁷⁵

1.6.2. Electrochemical approaches

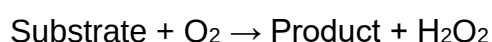
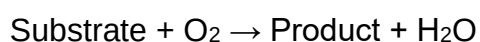
The need for rapid detection and monitoring in clinical, environmental and biodefense causes the new ways for development of alternative analytical devices based on the molecular recognition properties of macromolecular biomolecules and their electrochemical transduction. This type of electrochemical devices includes biosensors based on DNA, aptamer and protein. Electrochemical biosensors have a wide range application in food, pathogens, forensic applications, drug response measurement, cancer diagnosis and environmental issues.⁵⁰

The main idea of electrochemical biosensors is determination of electrical properties for information from biological systems and electrochemical applications use predominantly enzymes, antibodies, nucleic acids, cells and micro-organisms as a biological part.²⁶ An electrochemical biosensor can be obtained when the immunorecognition element (antibody, antigen, DNA or RNA) is immobilized on the surface of the transducer.⁶⁷

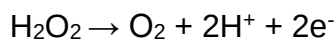
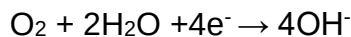
Typically, in bio-electrochemistry, a reaction will give a measurable current (amperometric), a measurable potential or charge accumulation (potentiometric) or a measurable alteration in the conductive properties (conductometric).¹⁶ Also, there are the other type of electrochemical detection techniques such as impedimetric in which impedance is measured, piezoelectric, and optic.⁵⁸ This section is for brief information about some types of biosensors and their working mechanism.

1.6.2.1. Amperometric biosensors

Amperometric type are a sub-class of electrochemical biosensors, and they are the most commonly reported class of biosensor.²⁶ They continuously measure current from the oxidation or reduction of electroactive components in a medium. The peak of the current measured over a linear potential range is proportional to the concentration of the analyte.²⁶ Bioactive materials that have been used in amperimetric biosensors are the oxidoreductases, also known as redox enzymes. Amperometric biosensors generally have the reactions;



According to the reactions above, consumption of oxygen can be measured by using Ag/AgCl reference electrode and Platine contrary electrode.



Amperometric electrodes are generally divided into three types;

First type of amperometric electrodes, are based on direct measurement of product from the reaction. Oxidant agents are not used in this type electrodes and electroactive product is measured directly. Second type of amperometric electrodes, oxidizing agents are used and allow for lower potential and less interference effect. Third type of amperometric electrodes, electron transfer is occurred and the main reaction between analyte and substrate can be measured directly. This kind of electrodes are developed by using conducting salts (see Figure 1.13).¹⁶

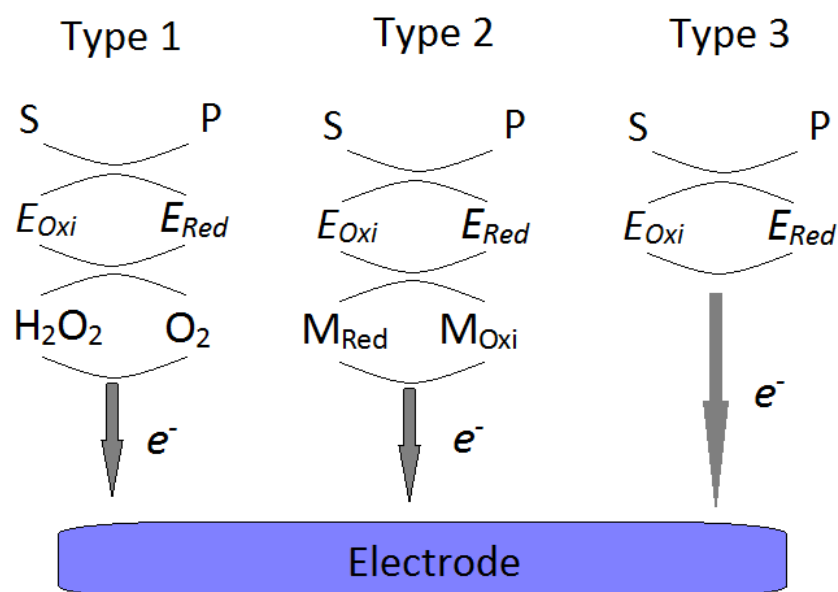


Figure 1.13. Types of amperometric electrodes (M_{Red} and M_{Oxi} are reduction and oxidation of agent, E_{Oxi} and E_{Red} are oxidation and reduction form of bioactive material, S and P are substrate and product)

An example of an amperometric device is for the electrochemical detection of cocaine using screen-printed cytochrome P450 2B4 based biosensors.⁵ Amperometric measurements were carried out for the characterization of the biosensors and limit of detection (LoD) value for the study was 0.12 ± 0.01 mM. The linear working range of the system is between 0.2 and 1.2 mM. In another study, butyrylcholinesterase was immobilized on Prusain Blue and LoD was found as 12 ng/mL(sarin) by using amperometric measurements.⁴ For detection of Amphetamine, metamphetamine, 3,4-methylenemetamphetamine, and 3,4-methylenedioxyamphetamine, urine samples were studied by using amperometry and linear working range was between 10^{-8} and 10^{-5} M. LoD was found as 3.3×10^{-9} M.⁷²

1.6.2.2. Potentiometric biosensors

Potentiometric measurement in this type of biosensors is the potential difference between an indicator electrode and a reference electrode. Potentiometric type sensors are used by using ion-selective electrodes to measure and determine the concentration of an analyte in gas or solution. The most common potentiometric devices are pH electrodes and ion-selective electrodes (F^- , I^- , CN^- , Na^+ , K^+ , Ca^{2+} , NH_4^+) (see Figure 1.14). According to Nerst-Donnan equation, the potential difference between the electrodes is proportional to the logarithm of the ion concentration.⁷⁶

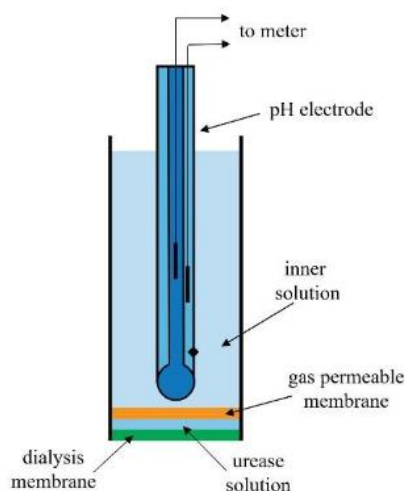


Figure 1.14. An example of potentiometric type chemical sensor.
(Adapted from chem.libretexts.org.)

$$E_{cell} = E_{cell}^0 - \frac{RT}{nF} \ln Q \quad (1.19)$$

According to Nerst-Donnan equation, E_{cell} represents the observed cell potential at zero current. E_{cell}^0 is a constant potential contribution to the cell, R is the universal gas constant and T is the absolute temperature in degree

Kelvin. n is the charge number of the electrode reaction, F is the Faraday constant and Q is the ratio of ion concentrations from anode and cathode.¹⁵

Low detection limits can be obtained with ion selective electrodes (ISE) and these limits are generally between 10^{-8} and 10^{-11} M. Potentiometric sensors are suitable for low concentration of the analyte in a sample.⁷ In a study, Dextromethorphan which is a drug of the morphinan class was detected by using potentiometric biosensor. The biosensor was fabricated by carbon paste electrode, PVC membrane electrode/ionic liquids. The linear working range was found as 10^{-5} to 10^{-2} M.⁷² Also, a biosensor was developed for chlorpromazine, which is classified as a low-potency typical antipsychotic. The electrode was fabricated with PVC membrane electrode/tetrabutylammonium tetraphenylborate, 2-nitrophenyl octyl ether. The potentiometric electrode showed the linear working range of 10^{-5} to 10^{-2} M.⁷²

1.7. Project Aims

Statistics have shown that the use of the ATS, particularly methamphetamine, have increased in recent years.²¹ Methamphetamine is the second most widely abused drug worldwide and therefore poses not only a major health risk to those who use it but also carries multiple social and economic burdens such as crime and health care resources.²¹ In order to keep up with the increased workload that has been attributed to the rise in methamphetamine, quick, robust analytical techniques have been desired. The overall aim of this project is to investigate the possible implementation of electrochemical analytical techniques for analysis of methamphetamine and compare the results with those of studies in the literature.

**Chapter 2- Electrochemical and
Electrogenerated Chemiluminescence
Studies and Characterisation**

Abstract

Modified electrodes can increase the rate of reaction over that observed at unmodified electrode surfaces. The primary benefit of these systems is that they combine the advantages of monolayer derivatised electrodes with those homogeneous catalytic systems, a high local concentration of catalytic sites despite the total amount of catalyst being small and easy separation of the reaction products from the catalyst and a three-dimensional dispersion of the reacting centres. A three-electrode configuration was used. A glassy carbon electrode (GC) was coated with $[\text{Ru}(\text{bpy})_3]^{2+}$ / Nafion film as working electrode. A platinum wire electrode and Ag/AgCl (saturated KCl) were used as counter electrode and reference electrode, respectively. The cyclic voltammograms (CVs) and electrochemiluminescent (ECL) response before and after the addition of MA in PBS were investigated on the $[\text{Ru}(\text{bpy})_3]^{2+}$ modified electrodes at a scan rate of from 100 to 500 mVs^{-1} . D_{ct} values observed are identical in this study ($D_{\text{ct-cathodic}}$ and $D_{\text{ct-anodic}}$). The results of D_{ct} are parallel to the results in the literature. This data shows that $[\text{Ru}(\text{bpy})_3]^{2+}$ centres can transport in the solution leading to conversion of the inactive Ru^{2+} centres into the mediating Ru^{3+} centres. The sensitivity of this sensor will ultimately be dictated by the rate at which the Ru^{3+} sites can be regenerated. Upon increasing the scan rate of the CV acquisition, it is typical to observe an increase in the anodic and cathodic peak currents. The system is linearly proportional to scan rate at 100 to 500 mVs^{-1} .

2. Introduction

For cyclic voltammetry, a voltammetric cell is needed which requires the presence of an electrolyte in solution primarily to complete the circuit but also to enable the migration of charged reactants and products. The time taken to complete the forward and reverse potential ramp is controlled by the scan rate and the total potential that is traversed. Despite the possibility of higher analysis rates, quantitative experiments using CV are restricted to 10^{-2} seconds.²⁷ During this ramp, the cell passes through initial potentials where no electrode reaction occurs and moving to potentials where a redox reaction occurs. Upon reaching the desired maximum potential, the scan direction is switched, and the potential is inversely ramped to the initial value.²⁷ The current of the working electrode is recorded by the potentiostat as a function of its potential measured against the reference electrode.²⁷

The reversible or irreversible behaviour of a CV system is determined by two factors known as the standard electrochemical rate constant (k^0) and the mass transport coefficient (m_T).⁴⁰ The mass transport coefficient can be defined as shown in Equation 2.1.

$$m_T = \frac{D}{\delta} \quad (2.1)$$

Where, δ is the diffusion layer thickness and D refers to the diffusion coefficient in cm^2s^{-1} . The relationship between k^0 and m_T determines the reversibility of the reactions as follows. If $k^0 \gg m_T$ then the redox reaction is reversible whereas if $k^0 \ll m_T$, the redox reaction is irreversible.⁴⁰

The peak current (i_p) for a reversible process can be defined by the Randles-Sevcik equation as shown in Equation 2.2.

$$i_p = 2.65 \times 10^5 n^{3/2} A D_{\alpha} \nu^{1/2} C \quad (2.2)$$

Where n is the number of electrons, A is the electrode area (cm^2), C is the concentration (mol cm^{-3}) and ν is the scan rate (V s^{-1}). Accordingly, the current is directly proportional to the concentration and increases with the square root of the scan rate. This equation is generally used for evaluation of D using cyclic voltammetry at relatively high scan rates ($> 50 \text{ mV/s}$).⁸³

For ideal reversible voltammetric behaviour, $i_{p,c}$ is equal to $i_{p,a}$. On the other hand, a variety of factors can influence the shape of a CV peak. The peak shape may differ from the ideal model due to background currents. Deviations are attributable to a subsequent chemical reaction that is triggered by the electron transfer. Other deviations can be that although the peaks may be approximately equal, the oxidation and reduction peaks do not centre on a single potential.⁸³

2.1. Experimental

2.1.1. Materials

$[\text{Ru}(\text{bpy})_3]^{2+}$ (Tris (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate) and Nafion 117 (per-flourinated ionexchange resin, 5% (w/v) solution in a mixture of alcohols) was purchased from Sigma-Aldrich. Phosphate buffer (PBS, 0.1 mol/L) was used to control the pH of aqueous solution (+) methamphetamine hydrochloride and DL-amphetamine were purchased from Sigma-Aldrich. All other reagents were of analytical reagent grade and ultra-pure water (Milli-Q plus, Millipore Inc.) was used throughout the experiments.

All electrochemical analysis was performed on a CH instrument (model 602E) using a standard three-electrode configuration. A glassy carbon electrode (GCE) was coated with $[\text{Ru}(\text{bpy})_3]^{2+}$ / Nafion film as working electrode. A platinum wire electrode and Ag/AgCl (saturated KCl) were used as counter electrode and reference electrode, respectively. All the potentials were measured and reported according to this reference electrode. The ECL experiments were carried using the CH instrument model 602E connected to a Hamamatsu H6780-20 PMT. The ECL cell was placed directly in front of the photomultiplier tube (PMT) which was enclosed in a light-tight box. A full description of the instrumentation employment can be found within our prior publications.^{17,20}

Before modification of GC electrode (d:3 mm), it was polished with 1, 0.3 and 0.05 μm aluminum solution on a polishing cloth, respectively and sonicated in water and washed with water. Then, it was dried.

2.1.2. Fabrication of $[\text{Ru}(\text{bpy})_3]^{2+}$ / Nafion ECL Sensor

The $[\text{Ru}(\text{bpy})_3]^{2+}$ /nafion film modified carbon paste electrode was fabricated by the following procedure: A 1 mM $[\text{Ru}(\text{bpy})_3]^{2+}$ solution was prepared in 50:50 EtOH:H₂O. 5 % w/v nafion was diluted to a concentration of 0.4 % w/v with 50:50 EtOH:H₂O. The final film concentration of 0.5 mM $[\text{Ru}(\text{bpy})_3]^{2+}$ and 0.2 % nafion was prepared by mixing the above solutions in a 1:1 (v/v) ratio. Once prepared the film was stored at 4 °C. The carbon working electrode modified with the film and left to air dry for 2 hours under darkness.

2.2. The detection of methamphetamine using $[\text{Ru}(\text{bpy})_3]^{2+}$ film

The cyclic voltammograms (CVs) and electrochemiluminescent (ECL) response before and after the addition of MA in PBS were investigated on the $[\text{Ru}(\text{bpy})_3]^{2+}$ modified electrodes at a scan rate of 100 mVs^{-1} . The modified electrode allows more $[\text{Ru}(\text{bpy})_3]^{2+}$ become $[\text{Ru}(\text{bpy})_3]^{3+}$ and an increasing ECL intensity can be seen, compared $[\text{Ru}(\text{bpy})_3]^{2+}$ solution phase works.

2.2.1. ECL measurement

The sensor fabricated was immersed into 0.1 M PBS in the presence of methamphetamine. The ECL measurement was performed at a potential range from 0.0 V to 1.6 V at scan rate of 100 mVs^{-1} with a conventional three-electrode system. Glassy carbon electrode fabricated with nafion and $[\text{Ru}(\text{bpy})_3]^{2+}$, a platinum wire and an Ag/AgCl (saturated KCl) electrode were used as working electrode, counter electrode, and reference electrode, respectively.

2.3. Results and Discussion

2.3.1. Electrochemical characterisation of ruthenium

The electrochemical behavior of 2 mM $[Ru(bpy)_3]^{2+}$ was examined in 0.1 M PBS using a glassy carbon electrode over a potential range 0.00 to 1.40 V vs Ag/AgCl at varying. Scan rates between $100 \leq v \leq 500 \text{ mVs}^{-1}$ over a potential range (Figure 2.1). In aqueous solutions, $[Ru(bpy)_3]^{2+}$ exhibits reversible one-electron oxidation (Pastore et al., 2006). The chemical reaction in the solution phase is;

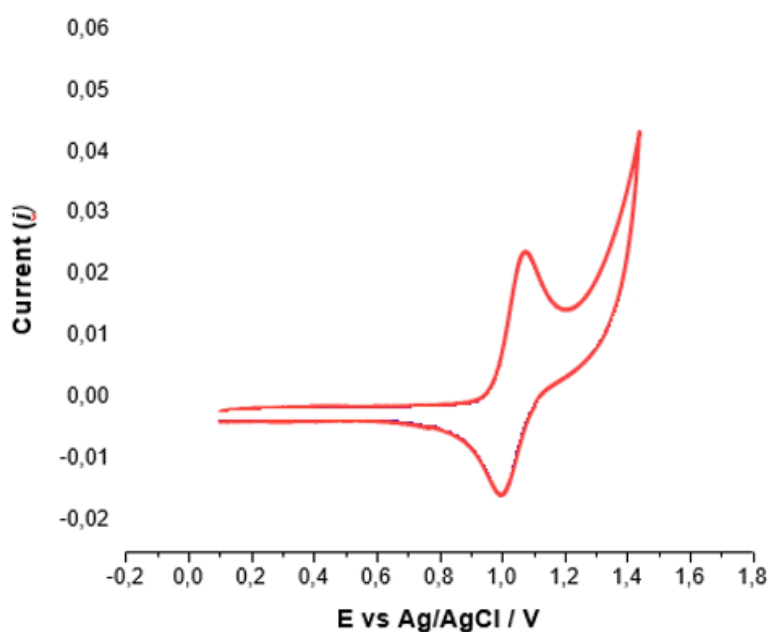
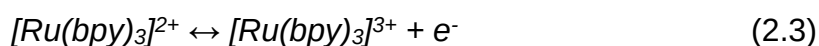


Figure 2.1. Typical cyclic voltammogram of 2 mM $[Ru(bpy)_3]^{2+}$ in 0.1 M PBS at a scan rate of 100 mVs^{-1} over the potential range of 0 to 1.4 V vs Ag/AgCl.

According to the Figure 2.1, the anodic peak potential at 1.05 V results from the oxidation of $[Ru(bpy)_3]^{2+}$ (see Equation 2.3). If a redox process is

reversible then the relationship between the anodic and cathodic peak currents (i_{pa}/i_{pc}) should equate to 1. This therefore indicated that the redox process may have been entirely reversible within this system.

2.3.2. Detection of MA via ECL

Based on the result from CVs, our system is sensitive to $[\text{Ru}(\text{bpy})_3]^{2+}$ and shows a reversible CV of $[\text{Ru}(\text{bpy})_3]^{2+}$. When MA is added into the media, an increase in oxidation peak (at about 1.15 V) was seen obviously. Our system is sensitive to MA and we can detect the changing in concentration of MA in the medium by CV.

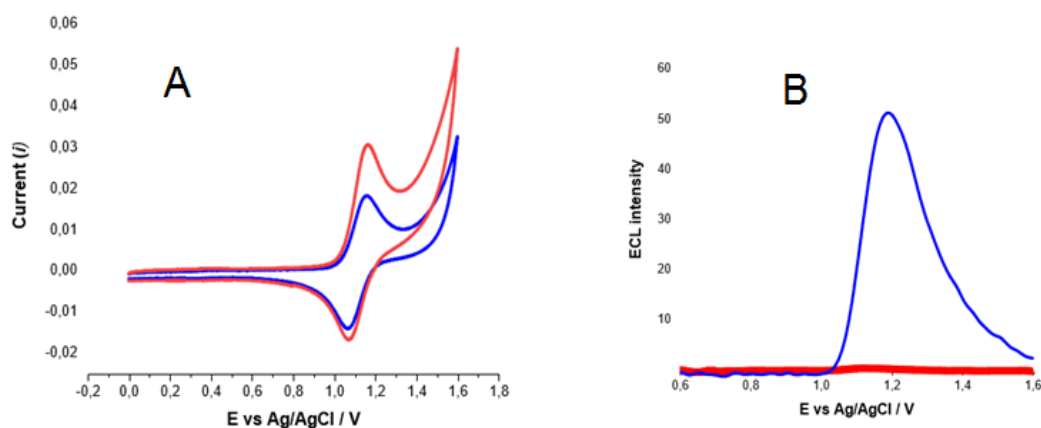


Figure 2.2. (A) Typical CVs of $[\text{Ru}(\text{bpy})_3]^{2+}$ without MA (blue line) and with MA (20 μM) (red line) in 0.1 M PBS scanned over the potential range $0.0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs. PMT was biased at 650 V. **(B)** ECL responses for 20 μM MA in 0.1 M PBS at pH 7.0 (blue line) and blank (red line)

Upon assessing the simultaneous ECL responses (see Figure 2.2), it is clearly evident that the presence of MA has an effect on the ECL behavior of the system which means like the oxalate system.⁵³ $[\text{Ru}(\text{bpy})_3]^{2+}$ is mediating the oxidation of methamphetamine which is resulting in the

formation of a $[\text{Ru}(\text{bpy})_3]^{*2+}$ excited state which emits photons of light upon relaxation. Based on the ECL results, it is obviously seen that $[\text{Ru}(\text{bpy})_3]^{2+}$ / MA mechanism follows the same pathway (co-reactant ECL) (see Chapter 1.4.2) as $[\text{Ru}(\text{bpy})_3]^{2+}$ / TPrA. Here, MA is used as co-reactant. $[\text{Ru}(\text{bpy})_3]^{2+}$ can be regenerated while MA is consumed during the electrochemical reactions (see the equations from 1.13 to 1.17).

Despite the lack of characterisation of the processes involved in this light emission, it is theorised that methamphetamine follows a similar mechanism to that of TPA co-reactant. The proposed mechanism for the ECL of $[\text{Ru}(\text{bpy})_3]^{2+}$ and methamphetamine is highlighted in Equation 3.1 to 3.4.

2.3.3. Diffusion Coefficient Investigation

In a diffusion-controlled system, the observed CV response is dependent upon the scan rate of the potential sweep as this determines how rapidly $[\text{Ru}(\text{bpy})_3]^{2+}$ can be oxidised to $[\text{Ru}(\text{bpy})_3]^{3+}$. Diffusion coefficient investigation is an important factor in ECL, as detection is based upon the production of $[\text{Ru}(\text{bpy})_3]^{3+}$ (see the equation 1.2 to 1.7). According to the Randles-Sevcik equation (see equation 1.2)

According to the equation, the current is directly proportional to the concentration and increases with the square root of the scan rate in diffusion-controlled systems. It is important to determine the rate of charge transport through the solution leading to the conversion of the inactive Ru^{2+} centres into the mediating Ru^{3+} centres. The rate of charge transport can be quantified by measuring the D_{ct} (called the charge transport of apparent diffusion coefficient) using CV. This parameter can be obtained from a plot

of i_p vs. $v^{1/2}$ under diffusion-controlled conditions (equation 2.4), usually favoured by higher scan rates. The upper scan rate is limited by the rate of heterogeneous electron transfer across the electrode layer interface.⁸³ In this study, these conditions are met $100 \leq v \leq 500 \text{ mVs}^{-1}$ and plots of peak current varied linearly with the square root of scan rate over this range, typical cyclic voltammetric scan rate dependency and Randles-Sevcik plots are shown in Figure 2.3.

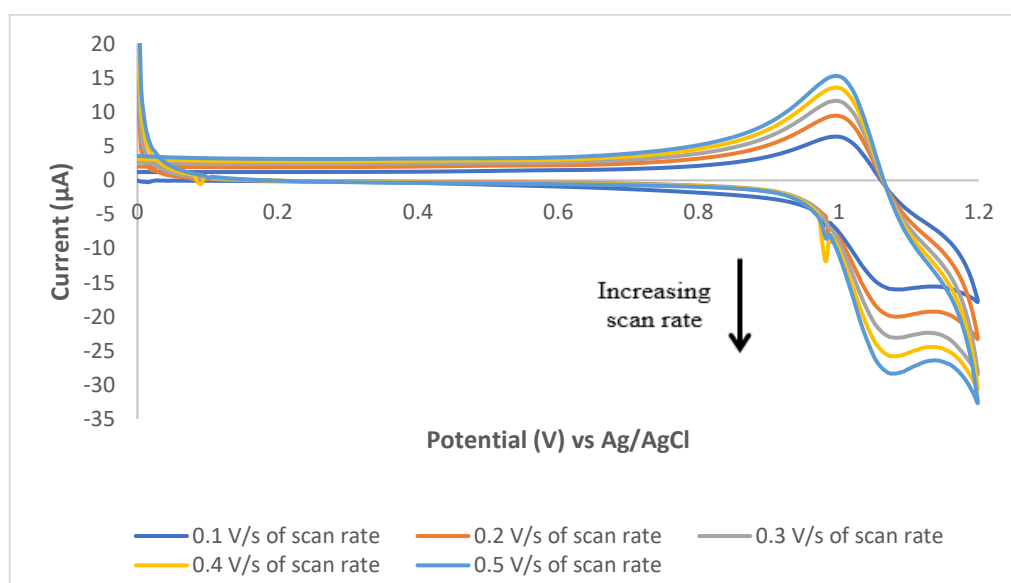


Figure 2.3. Cyclic voltammograms for 2 mM $[\text{Ru}(\text{bpy})_3]^{2+}$ in 0.1 M PBS at increasing scan rates from 100 to 500 mVs^{-1} as indicated by the arrow

Upon increasing the scan rate of the CV acquisition, it is typical to observe an increase in the anodic and cathodic peak currents (see the equation 2.4). This was observed in the $[\text{Ru}(\text{bpy})_3]^{2+}$ system and the linear relationship between the scan rate and the anodic and the cathodic peak current can be highlighted in Figure 2.3. As is typical of ruthenium solution phased systems, the system is linearly proportional to scan rate at slow scan rates. This proportionality can often be used to determine the system

which can generate $[\text{Ru}(\text{bpy})_3]^{3+}$. This system showed signs of reversibility and the electrode system can be used in 0.1 M PBS media for the further studies.

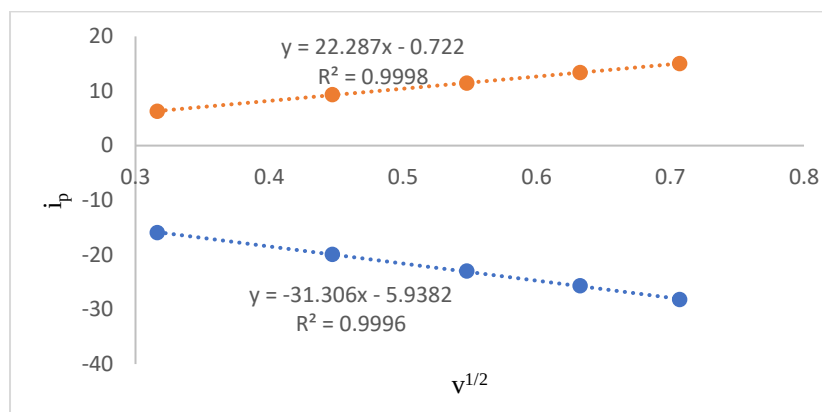


Figure 2.4. Effect of increasing scan rate on anodic and cathodic peak current monitored in 1 mM $[\text{Ru}(\text{bpy})_3]^{2+}$ in 0.1 M PBS. Several error bars are smaller in size than the associated data point.

Theoretically, diffusion coefficient is a proportionality constant between the molar flux due to molecular diffusion and the gradient in the concentration of the species. The higher the diffusion coefficient, the faster they diffuse into each other. As it can be seen from Figure 2.4 and Table 2.1, D_{ct} values observed are identical in this study ($D_{\text{ct-cathodic}}$ and $D_{\text{ct-anodic}}$). The results of D_{ct} are parallel to the results in the literature. This data shows that $[\text{Ru}(\text{bpy})_3]^{2+}$ centres can transport in the solution leading to conversion of the inactive Ru^{2+} centres into the mediating Ru^{3+} centres. The sensitivity of this sensor will ultimately be dictated by the rate at which the Ru^{3+} sites can be regenerated.

Table 2.1. Charge transport diffusion coefficient (D_{ct}) based on the results from Figure 2.3.

Dct(CV) Cathodic cm²s⁻¹	E⁰ V Vs Ag/AgCl	Dct(CV) Anodic cm²s⁻¹
7.43 E-09	1.03	3.77 E-09

2.3.4. Electrochemical characterisation of methamphetamine using CV

Despite the positive indications observed for methamphetamine detection using $[\text{Ru}(\text{bpy})_3]^{2+}$ in the solution phase, a method developed in this format would restrict the use of portable electrochemical instruments. An investigation into the potential use of immobilised $[\text{Ru}(\text{bpy})_3]^{2+}$ films on the working electrode surface was conducted in order to assess the possibility of circumventing this issue. Nafion polymers are widely used due to their resistance to chemical attack in harsh conditions such as elevated temperatures and the presence of strong oxidants. Nafion is favoured over alternative films for its high selectivity towards large hydrophobic cations such as $[\text{Ru}(\text{bpy})_3]^{2+}$.⁹⁷ This is due to the dissociable proton of the Nafion polymer which enables incorporation of electroactive cations from a contacting solution phase.⁹⁷

Nafion is one of the most commonly used cation exchange polymers. The structure of Nafion consists of an electrically neutral fluorocarbon

backbone (polytetrafluoroethylene) and a randomly tethered side-chain with a pendant ionic group, SO_3^- . The general chemical structure of Nafion is illustrated in Figure 2.7. Nafion has attracted particular attention due to its attractive electrochemical and thermal properties such as proton transportation, selective ion-exchange, efficient catalyst support and chemical stability.⁸⁵ Other fluorinated commercial membranes similar to Nafion were developed, such as, Hyflon, Flemion, 3P membranes, Gore-Tex and Gore Select, as well as Nafion-like membranes with $-\text{COOH}$ weak acid groups instead of $-\text{SO}_3\text{H}$, like Aciplex.⁸⁵ These membranes have chemical structures similar to that shown in Figure 2.5.

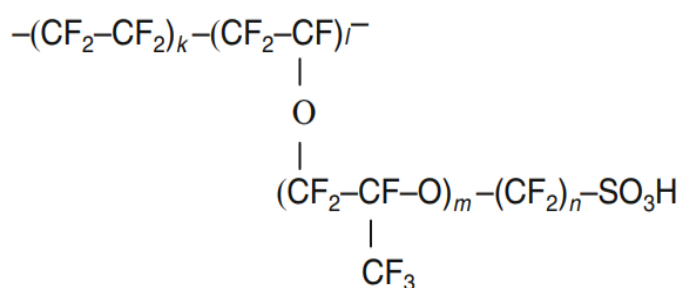


Figure 2.5. Chemical structure of Nafion and other perfluorinated sulfonic acid polymers

Ruthenium film solution was prepared by diluting 1 mM $[\text{Ru}(\text{bpy})_3]^{2+}$ in a 1:1 ratio with a solution of methanolic Nafion 117 (0.4% (w/v)). 5 μL of this methanolic solution was then applied to the surface of each GCE. In order to dry the solution onto the electrode surface, the electrodes were then placed in dark at room temperature for 2 hours. The GCE were then stored in the fridge until required for analysis. The voltammograms obtained in Figure 2.6.

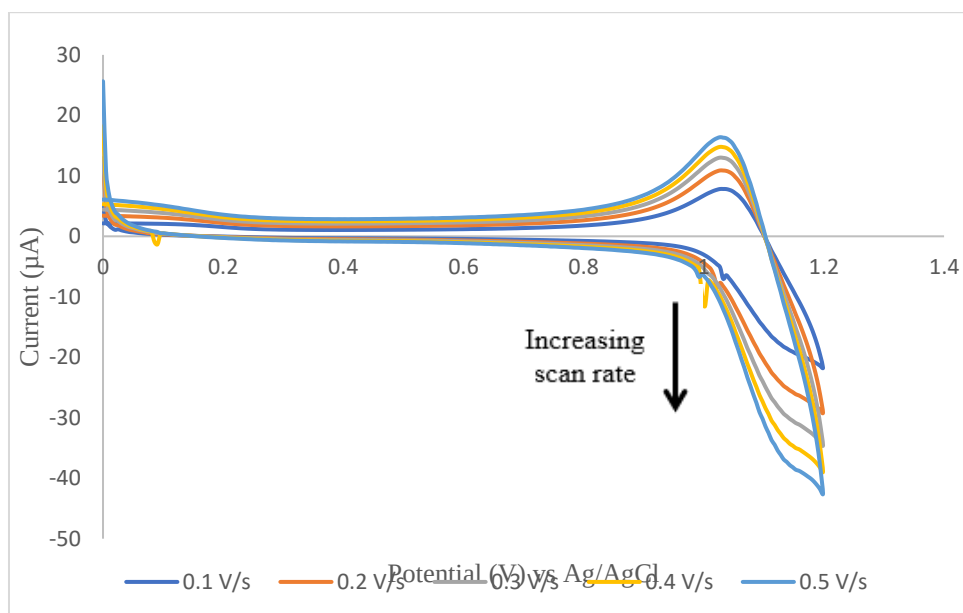


Figure 2.6. Cyclic voltammograms for 0.5 mM $[\text{Ru}(\text{bpy})_3]^{2+}$ film over a potential range of 0 to 1.2 V at increasing scan rates from 100 to 500 mVs^{-1} as indicated by the arrow

Upon increasing the scan rate of the CV acquisition, it is typical to observe an increase in the anodic and cathodic peak currents (see the equation 2.4). This was observed in the $[\text{Ru}(\text{bpy})_3]^{2+}$ system and the linear relationship between the scan rate and the anodic and the cathodic peak current can be highlighted in Figure 2.7. The system is linearly proportional to scan rate at 100 to 500 mVs^{-1} .

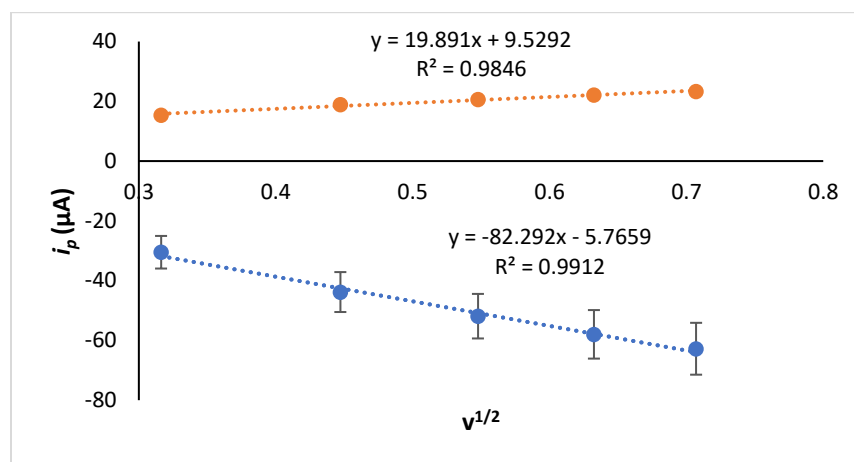


Figure 2.7. Effect of increasing scan rate on anodic and cathodic peak current. Several error bars in anodic peaks are smaller in size than the associated data point.

Table 2.2. Charge transport diffusion coefficient (D_{ct}) based on the results from Figure 2.6.

D_{ct}(CV) Cathodic cm²s⁻¹	E⁰ V Vs Ag/AgCl	D_{ct}(CV) Anodic cm²s⁻¹
8.33 E-5	1.091	4.8 E-6

According to the results from Table 2.2, $D_{ct}(CA)$ and $D_{ct}(CV)$ are identical in this study suggests that the density of the redox centres is uniform throughout the layer thickness. E^0 for the surface confined $Ru^{+2/+3}$ species was found as 1.091 V.

2.4. Conclusion

Modified electrodes can increase the rate of reaction over that observed at unmodified electrode surfaces. The primary benefit of these systems is that they combine the advantages of monolayer derivatised electrodes with

those homogeneous catalytic systems, a high local concentration of catalytic sites despite the total amount of catalyst being small and easy separation of the reaction products from the catalyst and a three-dimensional dispersion of the reacting centres.

There are less diffusional issues associated the use of films due to the immobilisation of $[\text{Ru}(\text{bpy})_3]^{2+}$ on the electrode surface and therefore a higher concentration of $[\text{Ru}(\text{bpy})_3]^{2+}$ molecules to interact with methamphetamine. So, the system showed a linear working range of lower concentration of methamphetamine compared with that of the unmodified electrode system. To sum up, despite of using less $[\text{Ru}(\text{bpy})_3]^{3+}$ molecule, methamphetamine was detected thanks to the benefits of modified electrode mentioned above.

Chapter 3- Electrochemiluminescence detection of methamphetamine in biological matrices

Abstract

This work presents an electrochemical detection method for the stimulants in the forensic samples using electrochemiluminescence (ECL). In this context, amphetamine type stimulant (methamphetamine (MA)) has been studied as coreactants in the $[\text{Ru}(\text{bpy})_3]^{2+}$ (where bpy is 2,2'-bipyridine) ECL system. This approach is developed based on a glassy carbon electrode modified with $[\text{Ru}(\text{bpy})_3]^{2+}$ /Nafion composite film. LoD, LoQ and linear working range for MA are studied currently. The ECL intensity was found to be concentrations over the range of 5×10^{-8} to 2.5×10^{-4} mol/L. LoD for MA is 1.94×10^{-10} mol/L. The regression coefficient is 0.9931 for the experiment. Our approach was applied in different medium such as saliva and human serum to detect MA. This technique is simple, rapid, selective and sensitive, and shows potential for the high-throughput quantitation of MA. The results show that the present electrochemical approach seems to provide a sensitive detection of MA in forensic applications.

3. Introduction

Amphetamine type substances (ATS) continue to be abused globally.⁹² Indeed around 27 million people worldwide are estimated to have used ATS, with the use of methamphetamine (MA) increasing in many countries including the US, Australia and the UK to name a few.⁹² Other ATS include methylenedioxyamphetamine, and methylenedioxyethyle-amphetamine (MDEA) as highlighted in Figure 1.3. MA is a potent central nervous system stimulant and is mainly used for recreational drug. There are two enantiomers of MA and they are levo-methamphetamine and dextro-methamphetamine. In pure amine forms, these enantiomers are equal in the mix and show racemic property. Dextro-methamphetamine is a much stronger central nervous system than levo-methamphetamine.⁶¹ Dextro-methamphetamine is generally in the form of a hydrochloride salt and this salt is called as 'ice' and 'crystal math'. The crystal math allows users to inhale the vapor of MA. This ability of hydrochloric form of MA enables higher and faster recreational effect.⁶¹

The determination of ATS is important within medicine fields due to the toxicological and addictive effects of ATS. The standard approach for determining the abuse and / or use of MA is usually completed by verifying the concentration of MA in biological samples such as urine. This is most often completed using immunoassays or chromatographic techniques which both require sample preparation before analysis.^{35,61} Immunoassays, in particular, are often the primary approach for the screening of illicit substances.^{35,36} However, specificity can be a challenge especially for determining which ATS is present.^{34,35} The need for rapid detection and monitoring in clinical, environmental and forensic fields has seen increased

research into this area with an emphasis on low-cost and reusable analytical devices. Electrochemical biosensors, including electrochemiluminescence (ECL) based sensors, have a wide range application in food, pathogens, forensic applications, drug response measurement, cancer diagnosis and environmental issues.^{51, 54, 65}

Electrochemical sensors, including electrochemiluminescence (ECL) can offer a viable alternative for applications within the forensic and clinical environments. The expansion of current developments within the field has seen the introduction of portable sensors which can be controlled via commercially available handheld devices or smartphone devices.^{6,23,25,32,54,57,81} One of the major advantages of ECL is the greater control over both the position and time of ECL reactions. ECL emission occurs only in the immediate vicinity of the electrode, which is beneficial for selectivity, multi-analyte detection using multiple electrodes, and sensitivity. Control over the timings of the reaction improves reproducibility.¹⁵ These advantages have resulted in the application of ECL across many diverse research fields, including food and water testing, clinical diagnostics and bio-warfare agent detection.^{69,79} There is also an increasing interest in oral fluid as alternative to plasma sample or urine in clinical and forensic cases because oral fluid can be collected directly and there is no need for an additional process when direct observation.³⁶ Based on the results in the literature, the samples of oral fluid show similar results to those of plasma and drug concentrations in oral fluid reflect concurrent plasma concentrations.^{36,50} Saliva is a mixture of secretions from three major and minor accessory salivary glands and commonly is referred to as saliva. Bacteria and food debris are also present, but the main and major

component is water.⁶⁵ Oral fluid is hypotonic compared to plasma and there is a considerable variety in ionic concentrations of resting and stimulated states. pH of oral fluid in healthy individuals is usually between 6.2 and 7.4. An adult may produce ~1000 mL/day of oral fluid with typical flows of 0.05 mL/min.⁵ The basicity of ATS, with pKa values of ~9.9 for MA⁶², facilitates the opportunity for detection within oral fluid as they are easily transferred to the saliva. The typical time frame for the detection of MA in saliva is ~1 hour following the ingestion of a 10 mg dose, highlighting the need for a rapid and portable detection approach. The concentrations observed are usually in the nM range in comparison to the plasma where typically μM concentrations are observed.

With ECL based sensors growing in popularity as alternatives for employment as point-of-care or at-scene devices alongside their proven ability to detect MA, further investigation into their viability for detection of MA in biological matrices is required. Within this contribution, we report on the development of a simple $[\text{Ru}(\text{bpy})_3]^{2+}$ ECL sensor for the rapid screening of MA within biological fluids including human serum and artificial saliva. This will be achieved with minimal sample preparation, providing a screening system capable of offering rapid results required by law enforcement and / or physicians without access to a dedicated laboratory facility.

3.1. Experimental

3.1.1. Materials and Reagents

Tris (2,2'- bipyridyl) - dichlororuthenium (II) hexahydrate, $[\text{Ru}(\text{bpy})_3]^{2+}$, (+) methamphetamine hydrochloride, phosphate buffered saline (PBS) tablets, human pooled serum, and 117 Nafion (~5 % (w/v) mixture of lower

aliphatic alcohols and water) were purchased from Sigma-Aldrich. 0.1 M PBS was used as the electrolyte. All other reagents were of analytical reagent grade and ultra-pure water (Milli-Q plus, Millipore Inc.) was used throughout the experiments. Artificial saliva (Bioténe® oral balance gel) and synthetic skin were commercially purchased and used as received.

All electrochemical analysis was performed on a CH instrument (model 602E) using a standard three-electrode configuration. A glassy carbon electrode (GCE) was coated with $[\text{Ru}(\text{bpy})_3]^{2+}$ / Nafion film as working electrode. A platinum wire electrode and Ag/AgCl (saturated KCl) were used as counter electrode and reference electrode, respectively. All the potentials were measured and reported according to this reference electrode. The ECL experiments were carried using the CH instrument model 602E connected to a Hamamatsu H6780-20 PMT. The ECL cell was placed directly in front of the photomultiplier tube (PMT) which was enclosed in a light-tight box. A full description of the instrumentation employment can be found within our prior publications.^{17,20}

3.1.2. Fabrication of $[\text{Ru}(\text{bpy})_3]^{2+}$ / Nafion ECL Sensor

The ruthenium film was prepared by drop casting 5 μL of a 1:1 1 mM $[\text{Ru}(\text{bpy})_3]^{2+}$ in 0.2 % Nafion 117/MeOH (1:3 v/v) solution onto the surface of a GCE (Figure 3.1). This was then allowed to dry for 2 hours in the dark. The GCE were then stored in the fridge until required for analysis.

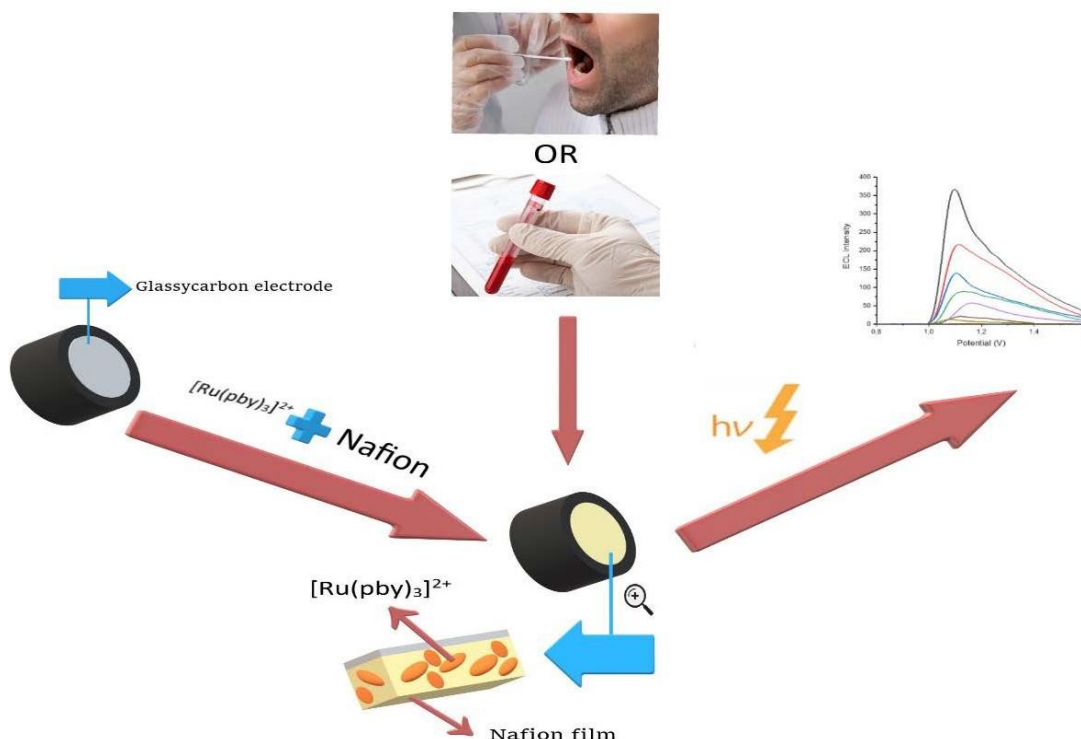


Figure 3.1 Schematic for the production of this sensor.

3.1.3. ECL measurement

The sensor fabricated was immersed into 0.1 M PBS in the presence of methamphetamine. The ECL measurement was performed at a potential range from 0.0 V to 1.6 V at scan rate of 100 mVs^{-1} with a conventional three-electrode system. Glassy carbon electrode fabricated with nafion and $[Ru(bpy)_3]^{2+}$, a platinum wire and an Ag/AgCl (saturated KCl) electrode were used as working electrode, counter electrode, and reference electrode, respectively.

3.1.4. Preparation of Biological Samples

All samples were prepared via spiking of the selected matrix with the required volume of ATS to give the desired concentration. Artificial saliva was commercially purchased. Due to viscosity of the artificial saliva, it was

diluted 5 times to observe optimum results. Results from “raw” artificial saliva were also recorded. Human pooled serum samples were stored at -80 °C and fully defrosted at room temperature prior to spiking and measurement. Human serum has strong background signal due to some interferences such as amino acids and proteins. To decrease the background signal. Dilution ratio of human serum was investigated, and optimum results was seen at the ratio of 1:3 (Human serum: PBS). Results obtained from non-diluted samples of pooled human serum were also recorded.

3.2. Results and Discussion

3.2.1. Detection of MA via ECL

Initial investigation of $[\text{Ru}(\text{bpy})_3]^{2+}$ was performed in 0.1 M PBS at pH 7.0 by using glassy carbon electrode. Analysis by CV was performed at the scan rate range of 100 mVs^{-1} over a potential range of 0.00 to 1.60 V vs Ag/AgCl (Figure 3.2).

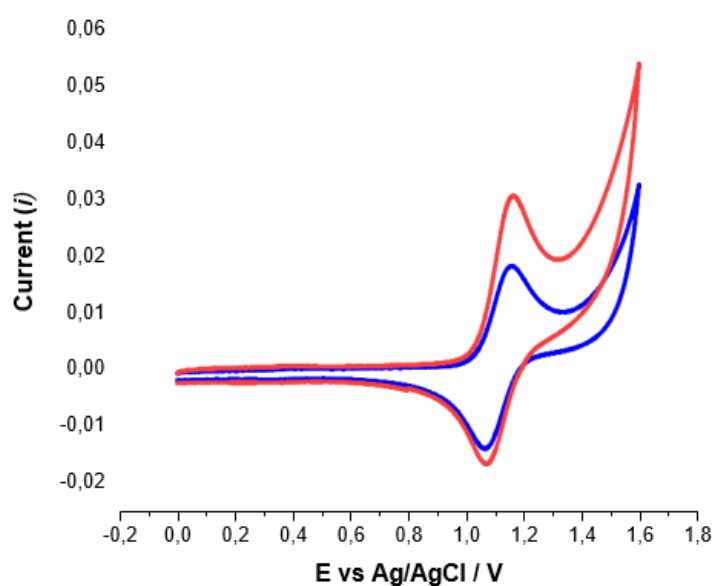


Figure 3.2 Typical CVs of $[\text{Ru}(\text{bpy})_3]^{2+}$ without MA (blue line) and with MA (20 μM) (red line) in 0.1 M PBS at pH 7.0 scanned over the potential range $0.0 \leq E \leq 1.6 \text{ V}$ vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

Based on the result from CVs, our system is sensitive to $[\text{Ru}(\text{bpy})_3]^{2+}$ and shows a reversible CV of $[\text{Ru}(\text{bpy})_3]^{2+}$. When MA is added into the media, an increase in oxidation peak (at about 1.15 V) was seen obviously. Our system is sensitive to MA and we can detect the changing in concentration of MA in the medium by CV.

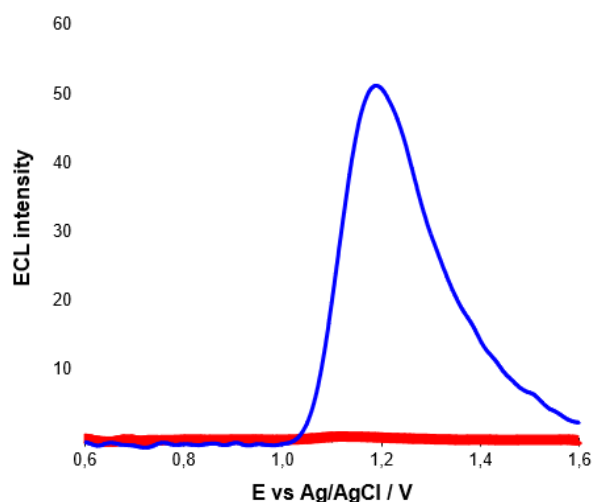
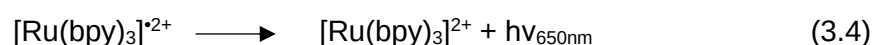
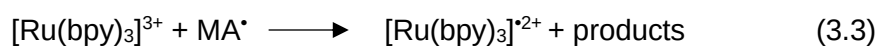


Figure 3.3 ECL responses for 20 μM MA in 0.1 M PBS at pH 7.0 (blue line) and blank (red line)

Upon assessing the simultaneous ECL responses (see Figure 3.3), it is clearly evident that the presence of MA has an effect on the ECL behavior of the system which means like the oxalate system.⁵³ $[\text{Ru}(\text{bpy})_3]^{2+}$ is mediating the oxidation of methamphetamine which is resulting in the formation of a $[\text{Ru}(\text{bpy})_3]^{*2+}$ excited state which emits photons of light upon relaxation.

Despite the lack of characterisation of the processes involved in this light emission, it is theorised that methamphetamine follows a similar mechanism to that of TPA co-reactant. The proposed mechanism for the ECL of $[\text{Ru}(\text{bpy})_3]^{2+}$ and methamphetamine is highlighted in Equation 3.1 to 3.4.



3.2.2. Analytical Parameters

To establish the adequacy of this sensor for both qualitative and semi-qualitative analysis, the analytical parameters were investigated. Previous work has shown that MA can be effectively detected in phosphate buffer⁵⁷ and that the optimal intensity for ECL production is close to the pKa of any amine. However, within this contribution the focus is on the performance at physiological pH ($\sim$ pH 7.0) reflecting the pH for the biological matrices investigated here. As shown in Figure 3.4, the ECL occurs at a potential of $\sim$ 1.15 V vs Ag/AgCl and increases with increasing [MA] over the concentration range 10 μ M to 25 mM. The response is linear over this range (Figure 3.4) with a LoD of 0.19 nM. Visual detection method was used for all LoD estimations during the present study.

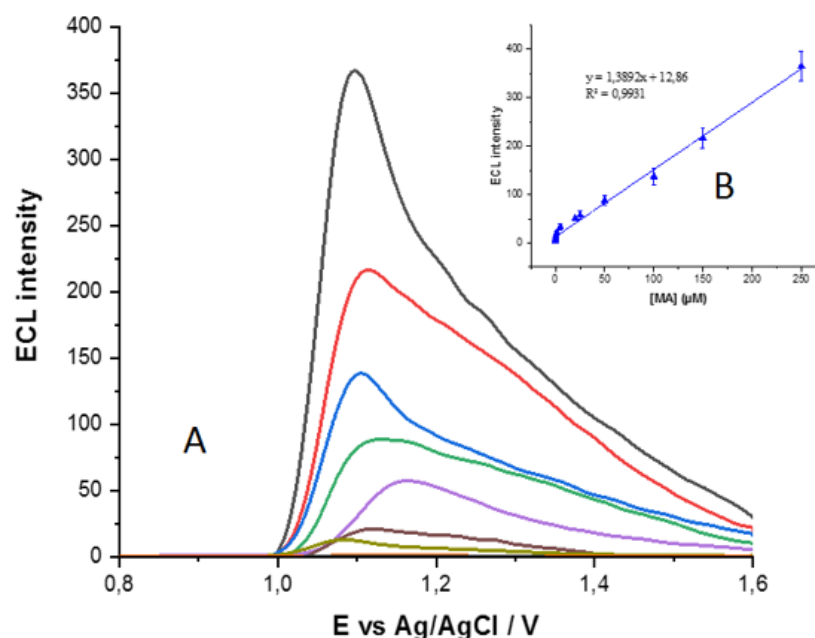


Figure 3.4 (A) Typical ECL response for increasing [MA] (0 to 250 μ M) in 0.1 M PBS at pH 7.0. (B) Limit of detection for MA in 0.1 M PBS at pH

7.0 scanned over the potential range $0.8 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs⁻¹. PMT was biased at 650 V.

This shows a slight improvement on previous ECL based sensors for MA detection.^{6,25,81} It is also possible that the detection limits achieved here could be improved further through the employment of a conducting polymer⁶³ rather than nafion to modify the electrode. ECL responses can also be obtained for other ATS⁵⁷, although these can often produce ECL signals of varying intensities and so it should be noted that this approach will not differentiate between the different ATS but can be utilised rather as a screening method for their presence.

Under the condition, ECL intensity of the developed ECL sensor was examined. ECL signals of MA were shown in Figure 3.4. The ECL intensity is linear with the concentration of MA in the range of 5×10^{-8} to 2.5×10^{-4} mol/L with the detection of limit of 1.94×10^{-10} mol/L. The regression coefficient is 0.9931 for the experiment. In the literature^{6,18}, most ECL sensors have LoD of 10^{-9} to 10^{-8} . Our system has very low detection limit and easy fabrication process. These features can make our system suitable for detection of real samples in forensic cases.

3.2.3. Biological fluid analysis

The biological fluid analysis for human serum and saliva were carried out under the same condition. Due to some amino acids and proteins in serum, high background signal was observed. a dilution step was used to find optimum dilution rate. The serum and saliva were diluted with 0.1 M PBS at pH 7.0. After the dilution process, three different concentrations of

MA were examined (100, 150 and 200 μM). % Recovery of saliva and human serum and standard deviations are shown in Table 3.1.

Table 3.1 % recovery of MA in diluted saliva and diluted human serum

[MA] μM	Saliva % Recovery	Human Serum % Recovery
100	97.91 ± 6.53	103.58 ± 9.33
150	98.88 ± 8.34	101.91 ± 8.30
200	100.04 ± 8.49	95.71 ± 6.74

Compared with the results from the literature above, our study has very easy process and easy application and is time saving. There is no need for extraction and pre-treatment of sample. we used a basic dilution just to decrease the background signal. Average of % recovery of our study in serum as well in saliva was very similar to that of PBS study. % Recovery studies show that our study is suitable for real samples when compared with the results in the literature.

3.3. Conclusion

Direct detection of amphetamine type stimulants (ATS) including methylamphetamine (MA) in biological matrices without the need for pre-treatment or extraction is a great challenge for forensic drug analysis. Electrochemical techniques, such as electrochemiluminescence (ECL), are promising tools for this area of analysis. This contribution focuses on the electrochemical and photochemical properties of $[\text{Ru}(\text{bpy})_3]^{2+}$ nafion

composite films and their subsequent use for the detection of ATS. The modified films were formed by direct interaction with the electrode surface without the need for surface modification or chain linkers. This is a major advantage for the fabrication of any sensor as it reduces the synthesis times resulting in more economically and cheaper production costs. This technique is simple, rapid, selective, and sensitive, and shows potential for the high-throughput quantitation of MA.

In conclusion, the present work shows voltammetric and the ECL behaviours of MA in $[\text{Ru}(\text{bpy})_3]^{2+}$ /nafion film system and this system has high LoD and % recovery rate in body fluids. The results show that our system can be used for real samples in forensic field. Also, the system can meet the need for fast and cost-effective detection of samples with high sensitivity in forensic field.

Chapter 4- Electrochemiluminescent Screening for Methamphetamine Metabolites

Abstract

The abuse of methamphetamine (MA) is to date detected and subsequently verified through the monitoring of MA and its metabolites within biological specimens. Current approaches require complex sample purification strategies alongside significant analysis time. Given the high prevalence of MA within the global drug market, there remains a need for rapid, portable and alternative screening approaches appropriate for direct detection within biological matrices for employment across the forensic and clinical environments. This contribution illustrates the use of an electrochemiluminescence (ECL) strategy for the screening of MA, amphetamine (AMP) and para hydroxy-methamphetamine (pOH-MA) for such applications. The sensing system showed ideal analytical performance with linear ranges at forensically relevant concentrations of 0.1 μM to 0.5 mM for MA, 10 μM to 1 mM AMP and 10 μM to 5 mM for pOH-MA, and superb detection limits of 74.6 nM, 6 μM and 82. μM for MA, AMP and pOH-MA respectively. Furthermore, the sensor was successful in the detection of MA, AMP and pOH-AMP within human pooled serum, artificial urine and saliva, without any prior purification strategies. Here a portable ECL sensor is detailed for the successful employment of the direct screening of these amphetamine type substances and their corresponding metabolites at clinically and forensically relevant concentrations within a range of biological matrices. This approach successfully represents a strong proof-of-concept, for a novel, simple and rapid screening method with significant potential for high-throughput screening of biological samples for drug metabolites, widening the avenues where ECL sensors could be employed.

4. Introduction

Methamphetamine (MA) remains amongst the highest abused illicit drugs within the world, with an estimated 29 million users globally.¹ MA belongs to a group of amphetamine type stimulants (ATS) which also includes amphetamine (AMP) and methylenedioxymethamphetamine (MDMA). ATS are now primarily considered a party drug, likely accounting for their widespread use. Yet frequent abuse of ATS is linked to an increased display of impulsive, violent and even homicidal behaviour and as such are still considered to present a significant threat to public safety.^{2,3}

As evident by their continued prevalence amongst drug users there still remains the requirement for appropriate screening methodologies to identify the abuse of ATS and allow law enforcement or physicians to rapidly determine the substance which has led to the consumers erratic, violent or life-threatening behaviour. A key focus of any screening strategies for employment within such environments, must place emphasis upon the application of the methodology toward a number of complex matrices including biological fluids; a common requirement faced within forensic and clinical practices. Biological fluid analysis presents its own unique complications, where accurate determination of a drug of abuse is necessary, despite the high number of metabolites present, which are often considered to be target analytes themselves.²⁻⁵

Immunoassays, which use antibodies for the targeted detection of a specific drug or metabolite, have seen wide employment across a number of fields including forensic practices and clinical toxicological screening.⁵⁻⁷ They are often the primary method employed for the screening of

substances of abuse, which can be attributed to their intrinsic advantages including, large scale screening facilitated through automation and rapid substance identification.^{4,5} What's more a number of commercial kits are available which currently offer home-testing or point-of-care analysis. However, immunoassays notably suffer from "false-positives" or "false-negatives" when drugs within the same class require identification, particularly within biological fluid analysis.⁴⁻⁹ Although these screening methods are the primary method employed for the detection of ATS, they fail to offer low-cost systems.

Electrochemical sensors, including electrochemiluminescence (ECL) based sensors, have become a viable alternative for applications within the forensic and clinical arenas. The growth in technology has facilitated the development of portable sensors which can be performed through commercially available handheld systems or even smartphone devices.¹⁰⁻¹⁶ Electrochemical sensors are primed to address the limitations faced by current immunoassay screening methodologies, with a reduction in cost, increased reusability, reduced instrument costs and minimal reagent consumption. Furthered though the proven ability of ECL sensors to offer direct drug detection within range of complex matrices, including biological fluids, without the requirement of any separation or purification strategies.¹⁶⁻²⁷

The detection of MA within biological fluids adopts a two-pronged approach, whereby identification of MA abuse is paired with detection of AMP as one of its primary metabolites.^{8, 28-30} Following ingestion, MA is readily absorbed into the blood stream, with excretion primarily through the renal system following hepatic metabolism, resulting in the primary

metabolites; AMP following the *N*-demethylation process and *para*-hydroxymethamphetamine (*p*-OHMA) following *para*-hydroxylation.³⁰ With typically, between 37-54 % of a MA dosage excreted via the renal system, urinary analysis is the primary biological fluid investigated for the identification of MA abuse.³ However, the basicity of ATS, with pKa values of 9.9²⁹ for MA and AMP, lends them well for detection within oral fluid, were they are observed to easily transfer from the blood stream to saliva of the consumer.²⁹ Typical concentration values reported for MA and AMP within urine, oral fluid and plasma range from as low as 0.12 μM to 20 μM for MA and 48 nM to 2.6 μM for AMP recorded across time intervals of $\sim$ 5 to 16 hours.^{8, 28, 29} As such, any sensing system developed must operate within these μM concentration regions within a range of biological fluids.

To date there is limited literature available on the electrochemical detection of ATS substances, despite the amine moiety previously demonstrating good electroactivity facilitating the electrochemical detection of a number of fellow amine containing compounds.^{17-20, 31-34} This limited investigation may be attributed to the high potential values required to initiate their oxidation, restricting the selectivity of voltammetric based detection, particularly within aqueous matrices. Previous studies have focus upon electrode modification to overcome the high oxidative potentials required. Of the ATS substances, MDMA, whose electroactivity is greater than its other ATS counterparts, is by far the most studied species to date.^{24,35} MA has seen significantly less investigation in comparison. 2016 marked the first use of a disposable electrode for the detection of MA, within undiluted saliva samples, through utilisation of *N,N'*-(1,4-phenylene)-dibenzenesulfoamide as a mediator.²⁵ Although this work was promising

for the detection of MA via portable electrochemical methodologies within biological matrices, the detection limits achieved lay outwith the clinically relevant range. Detection limits for MA have been improved via utilisation of ECL sensors, through employment of the traditional $[\text{Ru}(\text{bpy})_3]^{2+}$, facilitating mediated oxidation of the amine moiety within the species. Such employment achieved detection down to 4.0 fM.^{20,21} However, despite their promise these methodologies have displayed, to date, no electrochemical sensing system able to identify MA alongside its primary metabolites, AMP and *p*-OHMA, via a singular and direct detection strategy.

With ECL based sensors growing in popularity as viable alternatives for employment as point-of-care or at-scene devices, alongside their proven ability to detect MA and AMP, further investigation into their ability to simultaneously detect a drug of abuse alongside its metabolites within complex matrices will only stand to further their employment across a number of fields. Within this contribution we report the development of a basic $[\text{Ru}(\text{bpy})_3]^{2+}$ ECL sensor for the rapid screening of MA alongside its primary metabolites AMP and *p*-OHMA, within a variety of biological fluids, including human serum, artificial saliva and sweat. What's more we demonstrate the potential of the developed methodology for at-scene or point-of-care analysis through the negation of any sample purification procedures prior to analysis, providing a sensing system capable of offering the rapid answers required by law enforcement and physicians without access to a dedicated laboratory facility.

4.1. Experimental

4.1.1. Materials and Reagents

Tris (2,2'- bipyridyl) - dichlororuthenium (II) hexahydrate, $[\text{Ru}(\text{bpy})_3]^{2+}$, (+) methamphetamine hydrochloride, DL-amphetamine, phosphate buffered saline (PBS) tablets, human pooled serum, and 117 Nafion (~5 % (w/v) mixture of lower aliphatic alcohols and water) were purchased from Sigma-Aldrich. 0.1 M PBS was used as the electrolyte. *p*-hydroxy methamphetamine was purchased from Carbosynth Limited. All other reagents were of analytical grade and ultra-pure water (Milli-Q plus, Millipore Inc.) was used throughout the experiments. Artificial saliva (Biotène® oral balance gel) was commercially purchased and used as received.

4.1.2. Instrumentation

All electrochemical analysis was performed on a CH instrument (model 602E) using a standard three-electrode configuration. A glassy carbon electrode (GCE) was coated with $[\text{Ru}(\text{bpy})_3]^{2+}$ / Nafion film as the working electrode. A platinum wire electrode and Ag/AgCl (saturated KCl) were used as counter electrode and reference electrode, respectively. All the potentials were measured and reported according to this reference electrode. The ECL experiments were carried using the CH instrument model 602E connected to a Hamamatsu H6780-20 PMT. The ECL cell was placed directly in front of the photomultiplier tube (PMT) which was enclosed in a light-tight box. A full description of the instrumentation employment can be found within our prior publications.^{17-20, 37}

4.1.3. Fabrication of $[\text{Ru}(\text{bpy})_3]^{2+}$ / Nafion ECL Sensor

The ruthenium film was prepared by drop casting 5 μL of a 1:1 1 mM $[\text{Ru}(\text{bpy})_3]^{2+}$ in 0.2 % Nafion 117/MeOH (1:3 v/v) solution onto the surface of a GCE. This was then allowed to dry for 2 hours in the dark. The GCE were then stored in the fridge until required for analysis as outlined in scheme 1.

4.1.4. Preparation of Biological Samples

All samples were prepared via spiking of the selected matrix with the required volume of ATS to give the desired concentration. Artificial saliva was commercially purchased. Due to viscosity of the artificial saliva, it was diluted 5 times to observe optimum results. Results from “raw” artificial saliva were also recorded. Human pooled serum samples were stored at - 80 °C and fully defrosted at room temperature prior to spiking and measurement. Human serum has strong background signal due to some interferences such as amino acids and proteins. To decrease the background signal dilution ratio of human serum was investigated and optimum results seen at a ratio of 1:3 (Human serum: PBS). Results obtained from non-diluted samples of pooled human serum were also recorded.

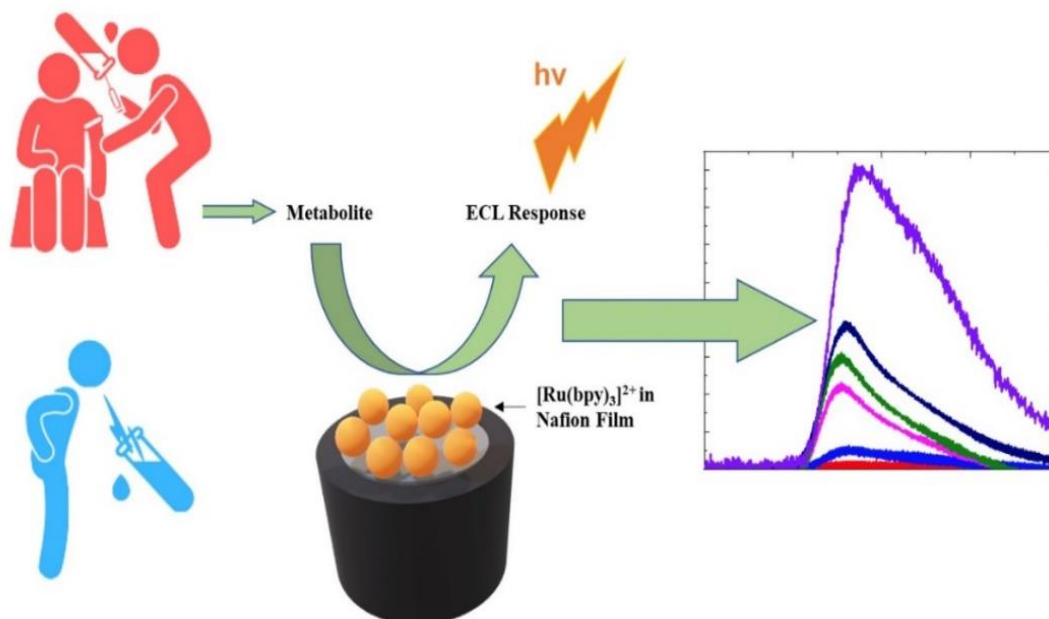


Figure 4.1. Schematic for biological sample analysis.

4.2. Results and Discussion

4.2.1. Detection of ATS via ECL

The approach utilised within this study was based upon the interaction of *p*OH-MA with a $[\text{Ru}(\text{bpy})_3]^{2+}$ complex which had been surface confined with a Nafion film on a GC electrode as previously reported.¹⁷⁻²⁰ The sensor was characterised and the same electrochemical behaviour as previously reported was observed. Amphetamine type substances (ATS) including methamphetamine and amphetamine, have been shown to produce an ECL signal upon interaction with a ruthenium (II) complex through the oxidative-reduction pathway.^{16,17}

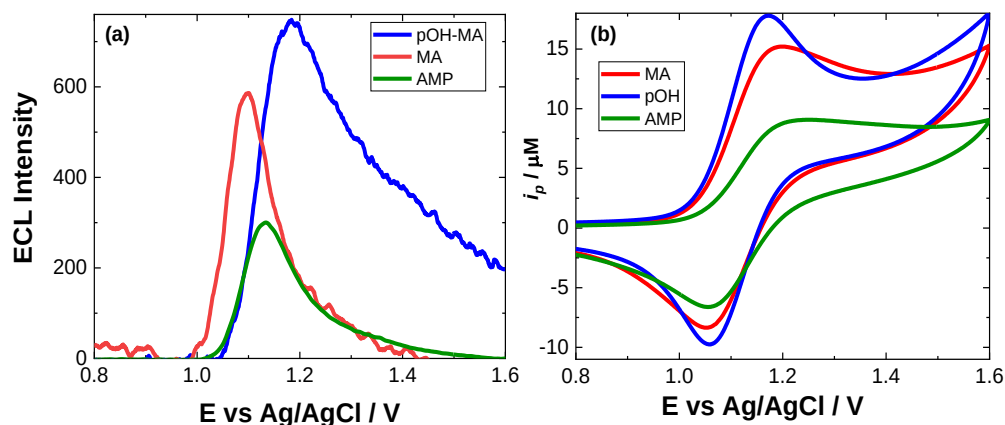


Figure 4.2. (a) Typical ECL response and (b) typical CV for 500 μM pOH-MA (blue), 500 μM AMP (green) and 500 μM MA (red) in 0.1 M PBS, pH 9. scanned over the potential range $0.8 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

Figure 4.2(a) shows the ECL response for the interaction of the surface confined ruthenium with MA, AMP and pOH-MA. Similar to the reaction of electrogenerated $[\text{Ru}(\text{bpy})_3]^{3+}$ with amino acids and structurally similar compounds, the reaction between pOH-MA and the Ru^{3+} undergoes a similar reaction process being sufficiently energetic to produce an ECL response.³¹ Direct electrochemical oxidation of pOH-MA was not observed over the concentrations examined within this study, as shown in Figure 4.3 and consistent with previous studies.²⁰

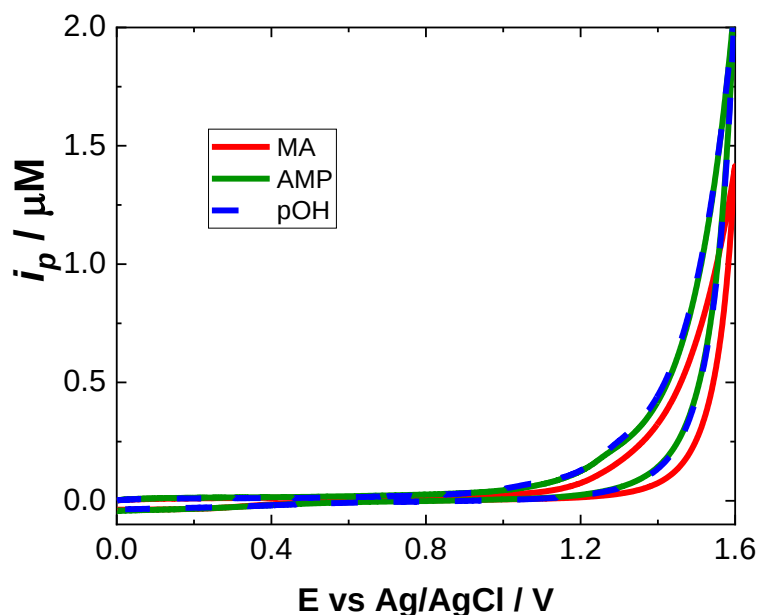
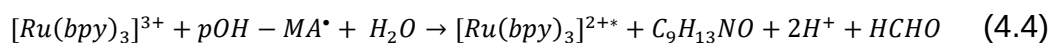
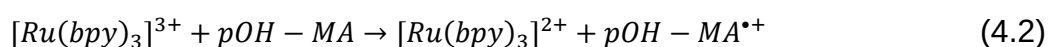


Figure 4.3. Typical CV for 150 mM MA (red line), AMP (green line) and pOH (blue dashed line) in 0.1 M PBS at pH 7.0 scanned over the potential range $0.0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} .

In the presence of $[\text{Ru}(\text{bpy})_3]^{2+}$, mediated oxidation of pOH-MA via the electrogenerated $[\text{Ru}(\text{bpy})_3]^{3+}$ species facilitated the production of an ECL emission through the mediated oxidative-reduction co-reactant ECL pathway with a maximum ECL intensity observed at ~ 1.15 V vs Ag/AgCl as shown in Figure 4.2(a). The ECL mechanism for pOH-MA is thought to follow a similar reaction process as that previously described for MA, amphetamine and other amino acids, as described.³⁷ When an anodic potential is applied on the system, $[\text{Ru}(\text{bpy})_3]^{2+}$ is oxidised at the electrode surface becoming $[\text{Ru}(\text{bpy})_3]^{3+}$ (Equation 4.1). This Ru (III) species then facilitates the oxidation of the pOH-MA via the mediated oxidation process, reported for a number of fellow amine species, which do not undergo direct oxidation at the electrodes surface. This process generates the unstable

radical cation, $pOHMA^{\cdot+}$ (Equation 4.2), which rapidly decomposes to form the highly reducing radical species $pOH-MA^{\cdot}$ (Equation 4.3). This free radical then undergoes an oxidation via the transfer an electron to $[Ru(bpy)_3]^{3+}$, forming the excited state ($[Ru(bpy)_3]^{2+*}$) (Equation 4.4), where excess energy is given off in the form of light (Equation 4.5).



Similarly, to other ATS, in the presence of $pOH-MA$, the onset of an ECL emission closely corresponds with the onset of oxidative current where the potential of Ru^{3+} is generated. Although no ECL emission is observed when $pOH-MA$ is absent, a typical voltammetric response for the $Ru^{2+/3+}$ redox couple is observed. ECL production for AMP, also a metabolite of MA, was examined and the ECL response is shown in Figure 4.2(b). This follows the same reaction mechanism and is consistent with previous investigations into the ECL co-reactant generation from ruthenium complexes with other ATS and amino acids.³¹ Direct electrochemical oxidation of AMP or $pOH-MA$, was not observed over the concentrations examined within this study, as shown in Figure S1. In the presence of $[Ru(bpy)_3]^{2+}$, mediated oxidation of both metabolites alongside the electrogenerated $[Ru(bpy)_3]^{3+}$ species

facilitates the production of an ECL emission through the oxidative-reduction co-reactant pathway. ECL mechanism with a maximum ECL intensity observed at ~ 1.15 V vs Ag/AgCl is observed for both metabolites. This is slightly shifted compared to that of MA, which shows an ECL maximum of ~ 1.10 V vs Ag/AgCl. However, for all the onset of the ECL response can be seen at ~ 1.00 V vs Ag/AgCl and therefore this difference in ECL maximum output cannot be utilised for discriminating between the different ATS compounds. The ECL signals obtained for these ATS clearly varies, each producing varying ECL intensities as well as slight differences in the ECL maximum potentials. This has been observed previously and is related to the R group attached to the amino group, with ECL intensity being highest for R groups with poor electron withdrawing characteristics.³¹ This also influences the pH behaviour observed within this contribution.

Given the similarities of the ECL observed for these ATS, it would not be possible to distinguish between them solely from their resultant ECL response. However, for a screening method, the detection of either MA and / or its metabolites would be sufficient. For both clinical and toxicological interpretation, an easily obtained and rapid “yes or no” would be greatly beneficial to either direct treatment or what and if further toxicological analysis is required. Therefore, despite the current inability to discriminate between these three ATS, this approach still meets the criterion of a screening method. For use within this field, analysis in biological matrices is key. The pH of these matrices can be quite different and so an understanding of the impact of pH on the ECL response is needed.

4.2.2. Evaluating the impact of pH on ECL analysis

As with previous studies on other structurally similar compounds, the ECL intensity observed is strongly dependent on pH.^{19 – 20, 31} The ECL intensity for ATS complexes is maximised at pH values near the pK_a of the N-terminal amine site. Indeed, experimental evidence has illustrated that the overall reaction efficiency is strongly influenced by the pH at which the analysis is undertaken in addition to the electron donating/withdrawing behaviour of the functional groups attached to the α -carbon of the amino acid moiety within the ATS structure. This is primarily due to the fact that the pH influences the dominant form of the molecule under different pH conditions as influenced by the molecules' dissociation mechanism. The ECL responses for MA at different pH values is shown in Figure 4.4.

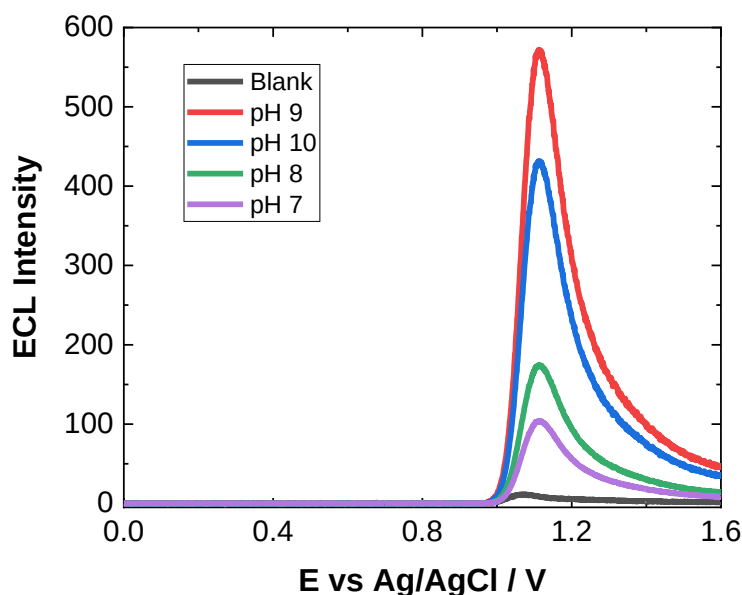


Figure 4.4. ECL response for 200 μ M MA in 0.1 M PBS at pH 7.0 (purple line), pH 8.0 (green line), pH 9.0 (red line), pH 10 (blue line) and a blank (black line) scanned over the potential range $0.8 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

A similar trend was observed within this study for pOH-MA with the maximum ECL intensity obtained at values close to its pKa.³⁸ As shown in Figure 4.4, the ECL intensity increases as the pH increases from 7, reaching a maximum intensity at ~pH 9. When the pH range was increased to values higher than pH 10, a significant background signal was observed. This is due to formation of the excited state from a secondary reaction between $[\text{Ru}(\text{bpy})_3]^{3+}$ and OH^- . The normalised ECL intensity is defined as the ratio of signal intensity ($I_{\text{pOH-MA}}$) to background intensity (I_{BKG}). ECL signals were clearly observed across the entire pH range evaluated to varying degrees. This is extremely relevant when considering analysis in a variety of biological matrices where the samples will have different pH values. In this context, MA and AMP were also studied to interrogate their pH dependence for ECL production.

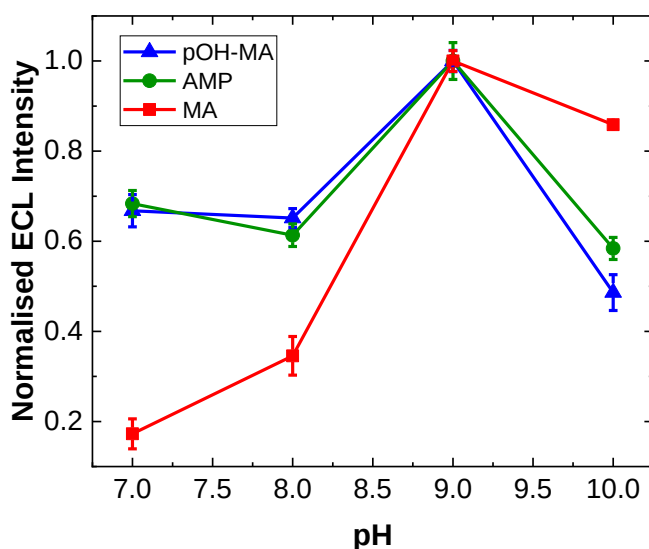


Figure 4.5. Typical trend for the dependence of the ECL intensity on pH of the electrolyte for pOH-MA (blue triangles), AMP (green circles) and MA (red squares). The ECL intensity was background corrected and normalised based on the maximum ECL response for each ATS for clarity (Error bars represent standard deviation obtained from triplicate results).

The highest ECL intensity of MA, AMP and pOH-MA were observed at pH 9.0 (pKa of MA: 10.1, pKa of AMP: 10.5, pKa of pOH-MA: 9.8). (Figure 4.5) These results are supported by previous work which illustrate the increased difficulty to oxidise amine species at low pH values, where their protonated and hence not electrochemically active form is dominant.^{17,18,19,20,39}

4.2.3. Analytical Parameters

To ensure sensor performance was adequate for both qualitative and semi-quantitative analysis, the analytical parameters for this ECL based sensor were evaluated. As mentioned, while pH 10 may provide the greatest ECL response, pH 9 represents a much greater signal to noise (S/N) ratio and is therefore more discriminating. However, to evaluate the response over different pH values, reflecting the range of pHs observed for biological matrices investigated within this study, calibrations were conducted at pH 7 and 10.

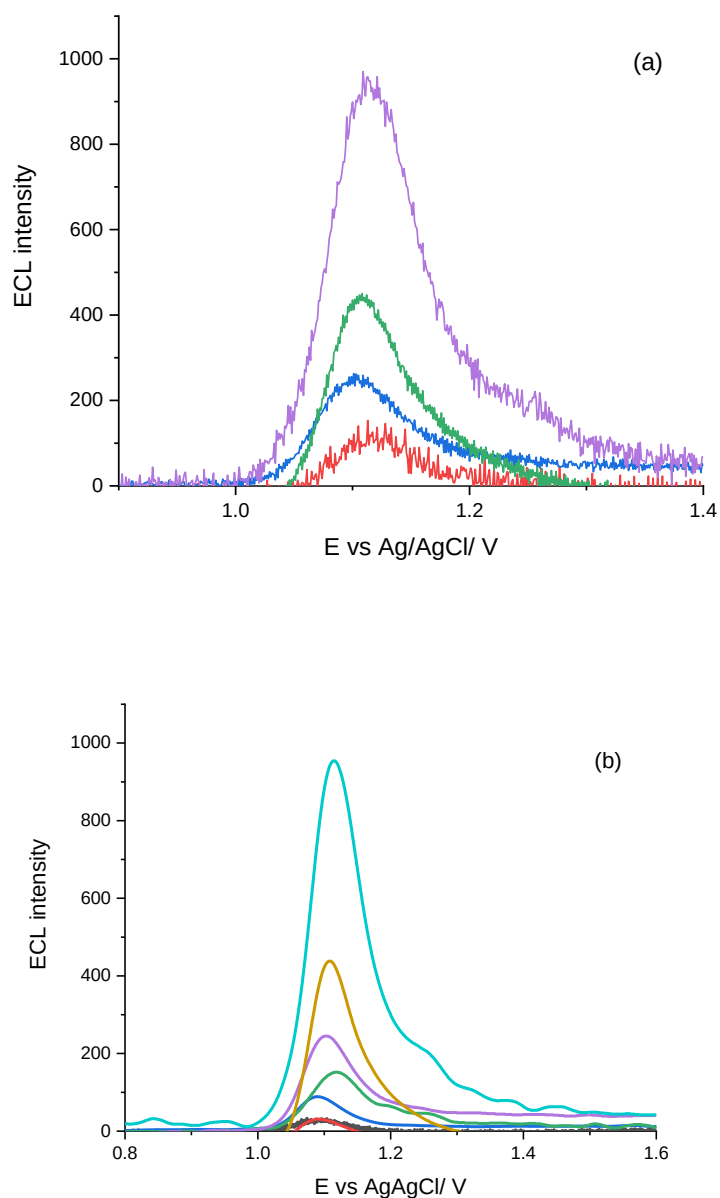


Figure 4.6. (a) ECL response for increasing [MA] in 0.1 M PBS at pH 7.0. (b) ECL response for increasing [AMP] in 0.1 M PBS at pH 7.0 scanned over the potential range $0.8 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

Linear coefficients (R^2) of 0.9971, 0.9967 and 0.9977 were obtained for the pH values investigated as shown in Table 4.1 and Figure 4.8(a). The limit of detection (LOD) at each of the pH values was also calculated based

upon the lowest concentration were an observable signal was still distinguishable at a signal to noise ratio of 3, summarised in Table 4.1.

The influence of *p*OH-MA, AMP and MA concentrations on ECL intensity was evaluated over the concentration range 0.1 μ M and 0.5 mM, consistent with the expected values that would be observed within biological fluids after ingesting MA⁸ and comparable to previous studies in optimised sample matrices, as shown in Figure 4.8(a)^{9 – 11}. Figure 4.8(b) illustrates the increase in the ECL response, taken at the peak maximum for each analyte, as the concentration of *p*OH-MA increases. This trend is similar for both MA and AMP (see Figures 4.6) at pH 10 as well as at pH 7 (see Figure 4.7), as well as other compounds with comparable amine functional groups. LOD for the biological samples are slightly higher, as shown in Table 4.1, due to the interference effects of natural amino acids. Despite this, they are still within the forensic and toxicologically relevant range. The forensically relevant ranges for MA and AMP are 0.12 to 20 μ M and 48 to 2.6 μ M respectively. The range for MA is therefore appropriate for relevant forensic samples, however, improvements are needed for AMP and *p*OH.

Table 4.1. Analytical results of this study under ideal conditions of pH 9
0.1 M PBS and the results from the literature

<i>ECL Approach for ATS</i>	<i>LoD</i>	<i>LoD in Biological Matrix</i>	<i>Linear range</i>
<i>MA - this Study</i>	74.6 nM	0.2 μ M serum 0.5 μ M urine 10 μ M saliva	0.1 μ M to 0.5 mM
<i>AMP – this Study</i>	1 μ M	6 μ M serum 2 μ M urine 10 μ M saliva	10 μ M to 1 mM
<i>pOH-MA – this Study</i>	82.8 nM	4 μ M serum 4 μ M urine 10 μ M saliva	0.1 mM to 5 mM
MA - Multiwall carbon nanotube/ionic liquid composite electrode ⁹	8.0 nM		10 nM to 80 μ M
MA - Glassy carbon electrode modified with [Ru(bpy) ₃] ²⁺ - doped silica nanoparticles/ Nafion composite film ¹⁰	26.0 nM		0.1 μ M to 10 μ M
MA - ECL sensor organically modified with silica film ¹¹	0.2 μ M		0.5 μ M to 1 mM

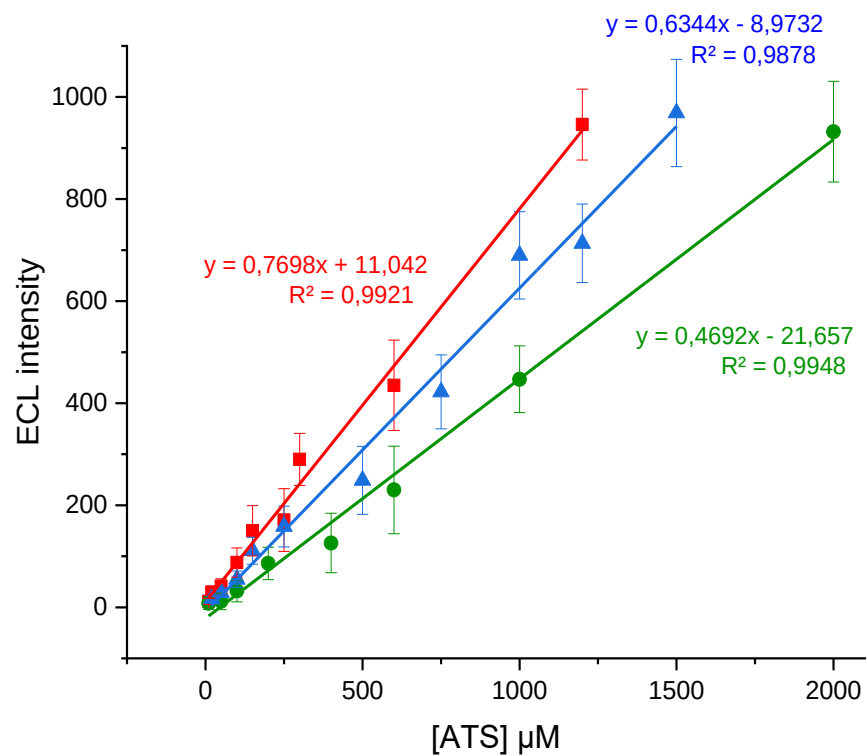


Figure 4.7. (a) Typical trend of maximum ECL signal against [ATS] at pH 7.0 for MA (red squares), AMP (green circles) and pOH-MA (blue triangles).

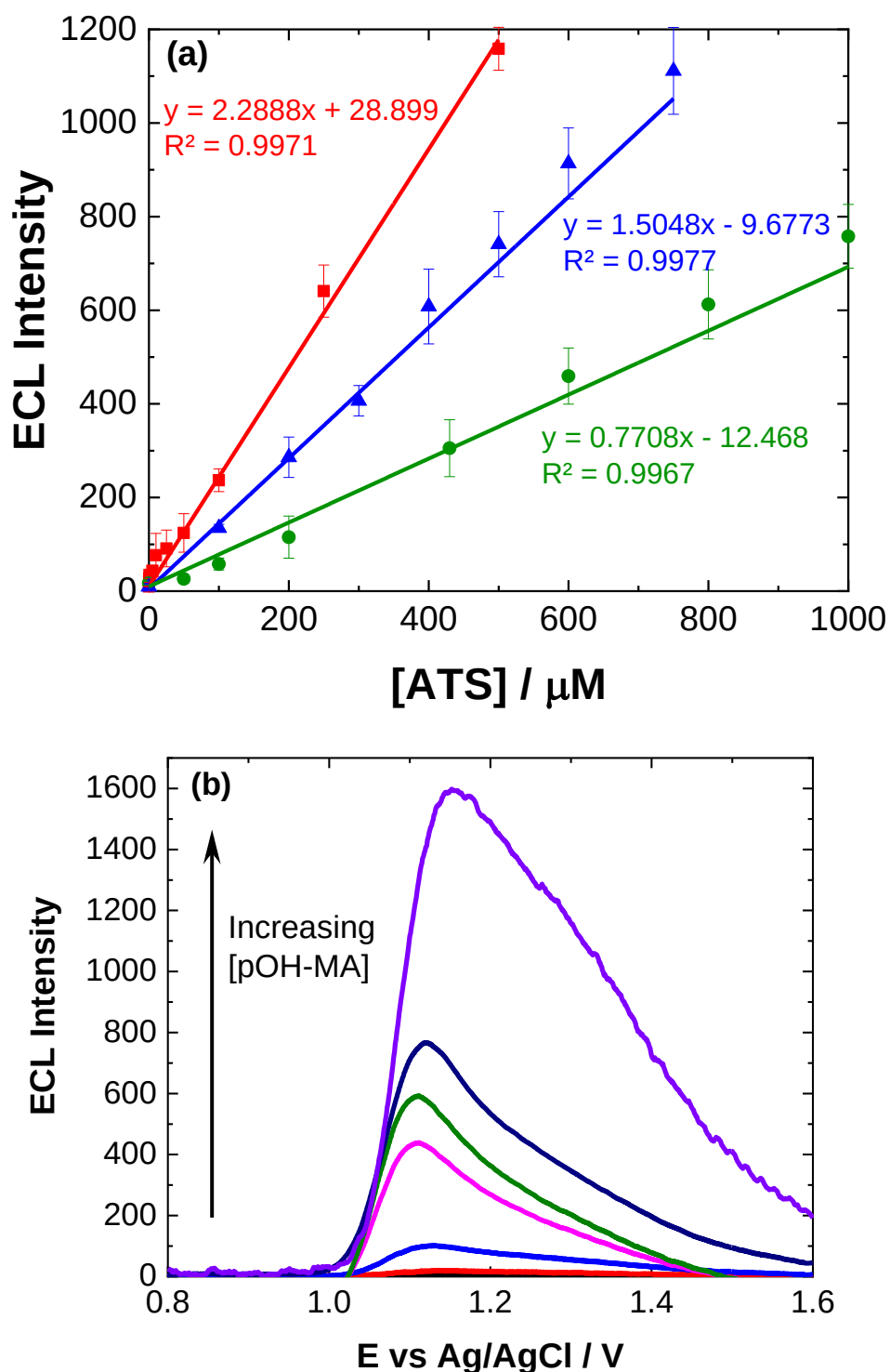


Figure 4.8. (a) Typical trend of maximum ECL signal against [ATS] at pH 9.0 for MA (red squares), AMP (green circles) and pOH-MA (blue triangles). (b) Typically, ECL response for increasing [pOH-MA] in 0.1 M PBS at pH 9.0 scanned over the potential range $0.8 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V (Error bars represent standard deviation obtained from triplicate results).

4.2.4. Biological Fluid Analysis

To evaluate the capacity of our approach for toxicological analysis, a range of biological fluids typically used for ATS analysis were examined. The biological matrices chosen included artificial saliva and pooled human serum. These samples are utilised to illustrate the feasibility of this approach for screening within complex biological matrices which will have numerous interferences. We report here for the first time, a method for screening of ATS in biological samples. In the literature, there is very limited study about *p*OH-MA in body fluids by ECL and electrochemical methods. Therefore, the results of this study were compared to standardised laboratory methods for ATS analysis, namely, LC-MS and GC-MS.³⁶

4.2.4.1. Artificial Saliva

To determine the practicality of our ECL method, we first used saliva as biological sample analysis. Saliva is a practical alternative to blood samples for forensic analysis. The clear advantage for portable analysis, is that unlike blood samples, saliva samples are much easier to obtain and does not require the degree of sample preparation often required for blood or serum samples. In the initial analysis, the direct detection of the ATS within artificial saliva was evaluated.

Previous studies using screen-printed electrodes illustrated that an ECL response can be obtained from directly in saliva, although it is advantageous to dilute it due to the issues surrounding its viscosity.⁴² However, when using conventional GC electrodes, the issue of viscosity was more pronounced and an ECL response could not be obtained, despite the fact that no ECL response was obtained from the blank artificial saliva

sample before spiking. Indeed, after dilution, as seen in Figure 4.9(a) the blank response is so low as to be indistinguishable from the baseline. Upon dilution with 0.1 M PBS (pH = 9.0) in a ratio 1:5 (v/v), an ECL response from the spiked artificial saliva is clearly visible as shown in Figure 4. The concentration of the ATS reported is that prior to dilution. This figure also highlights that the response from control or blank artificial saliva alone does not produce any significant interferent. The rational for achieving an increased response after dilution is attributed to the lowering of the viscosity of the sample therefore allowing sufficient kinetics to take place facilitating the production of the ECL response and to a lesser extent, to the shift in pH from ~7.4 to the optimal pH for ECL detection of *p*OH-MA at ~ pH 9.0.

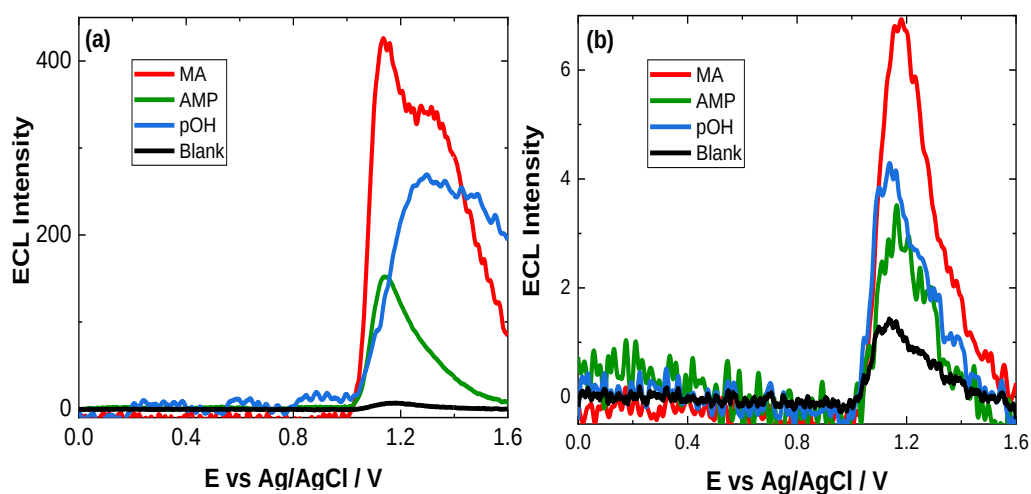


Figure 4.9. (a) ECL of 200 μ M MA (red), 200 μ M AMP (green), 200 μ M pOH-MA (blue) and a blank (black) in saliva after a 1:5 dilution (v/v) with 0.1 M PBS (pH = 9.0). (b) ECL of 10 μ M MA (red), 10 μ M AMP (green) and 10 μ M pOH-MA in saliva after a 1:5 dilution (v/v) with 0.1 M PBS (pH = 9.0) scanned over the potential range $0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

As evident from Figure 4.9(a), at high concentration of drug, a secondary peak is observed. This is likely due to diffusional issues within this quite viscose sample matrix. However, this is not an issue at the concentrations expected to be obtained from saliva samples (Figure 4.9(b)).

4.2.4.2. Serum Analysis

The experiment for human serum was carried out under the same condition. When ECL intensity of human serum was studied, very high background signal was observed (see Figure 4.10 and 4.11) due to the

presence of some amino acids and proteins in human serum which was expected.¹⁷

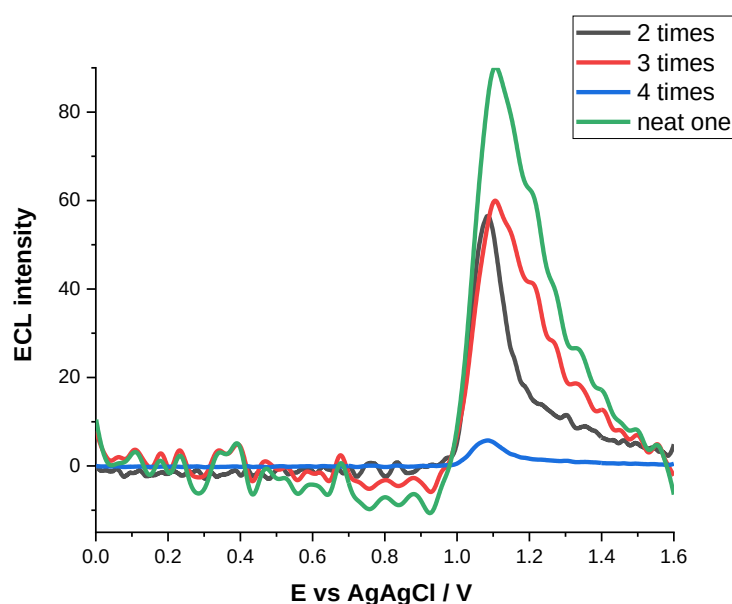


Figure 4.10. Background signals of serum neat blank and blanks after dilution (v/v). Green line is neat serum signal, black line is 2 times dilution (1:1), red line is 3 times dilution (1:2) and blue line is 4 times dilution (1:3) with 0.1 M PBS (pH = 9.0) scanned over the potential range $0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V. Signal to noise improved with reduced viscosity.

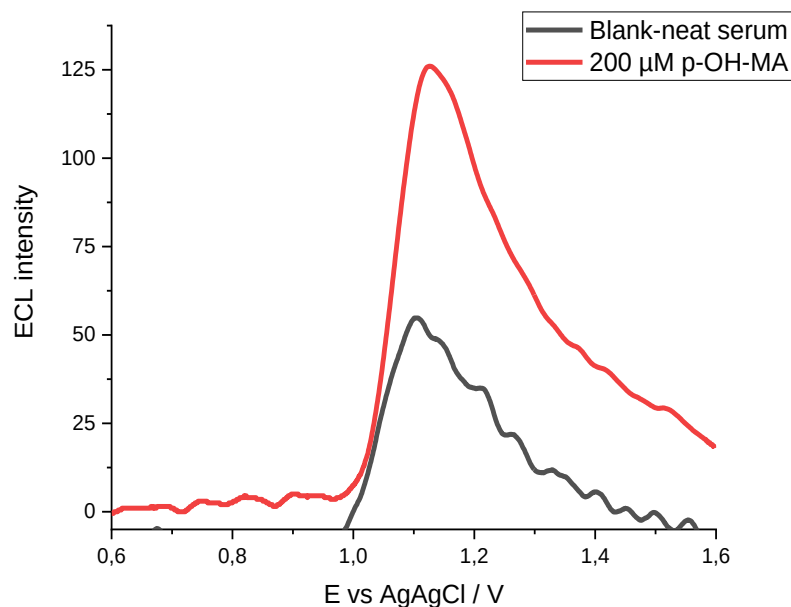


Figure 4.11. Background signal of neat serum (black line) and ECL signal of 200 μM *p*-OH-MA in neat serum (red line) over the potential range $0 \leq E \leq 1.6 \text{ V vs Ag/AgCl}$ at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

To find the optimum ECL intensity for human serum, a dilution step was applied (see Figure 4.12). The serum was therefore diluted in a 1:3 ratio 0.1 M PBS (pH 9).

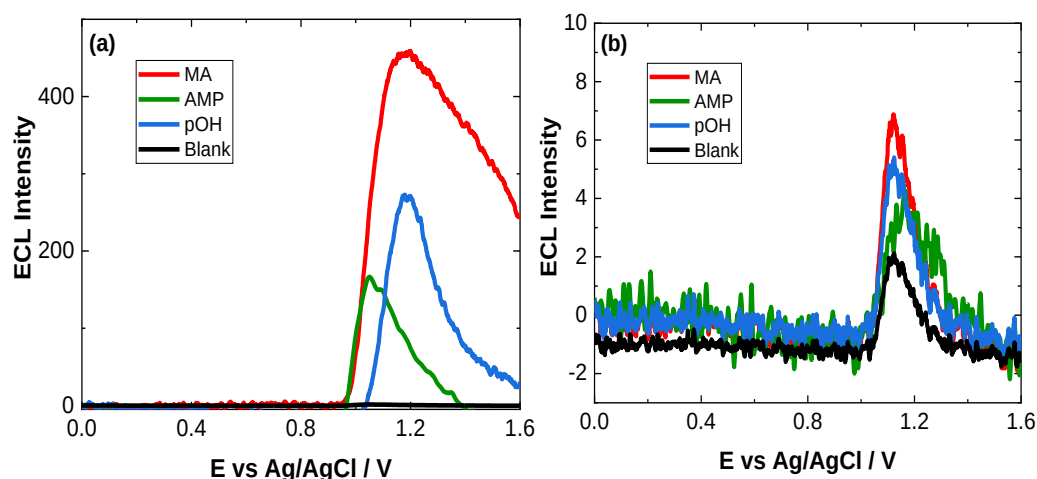


Figure 4.12. (a) ECL of 200 μM MA (red), 200 μM AMP (green), 200 μM pOH-MA and a blank (black) in human serum after a 1:3 dilution (v/v) with 0.1 M PBS ($\text{pH} = 9.0$) scanned over the potential range $0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V. (b) ECL of 0.2 μM MA (red), 6 μM AMP (green) and 4 μM pOH-MA in human serum after a 1:3 dilution (v/v) with 0.1 M PBS ($\text{pH} = 9.0$) scanned over the potential range $0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

Chromatographic analysis of p-OH-MA by LC-MS-MS achieved recovery rates of between 85 – 90 % for spiked blood / serum samples in the μM range.^{31,32} The % recovery rates achieved here are consistent with these previous reports, however the approach described here would be effective as a portable, rapid screening method at crime scenes or as a point-of-care device. This is primarily due to the simple process, particularly for saliva and urine samples, which negates the need for sample preparation. Indeed, if needed this approach could be implemented without any pre-treatment, although lower recovery rates would be achieved.

According to the results from the experiments, we report pH effect is very important in ECL intensity and ECL detection of MA and its main metabolite by a modified electrode system with $[\text{Ru}(\text{bpy})_3]^{2+}$ and nafion film can be done in body fluids.

4.2.4.3. Urine Analysis

The experiment for artificial urine was carried out under the same condition. When ECL intensity of the urine was studied, very high background signal was observed and similarly to the process applied for human serum was employed, a dilution step was incorporated. Urine samples were therefore diluted with 0.1 M PBS in a 1:4 v/v ratio (see Figure 4.13).

% Recovery of *p*OH-MA, MA and AMP in diluted human serum (1:3), artificial saliva (1:5) and urine (1:4) was investigated and the maximum ECL intensities compared to that of the ATS in 0.1 M PBS. In the experiment, three different concentrations were used (150, 200 and 250 μM). % Recovery ratio of each body fluid was shown in Table 4.2. Based on the results, our system is working in body fluids and has high recovery ratio.

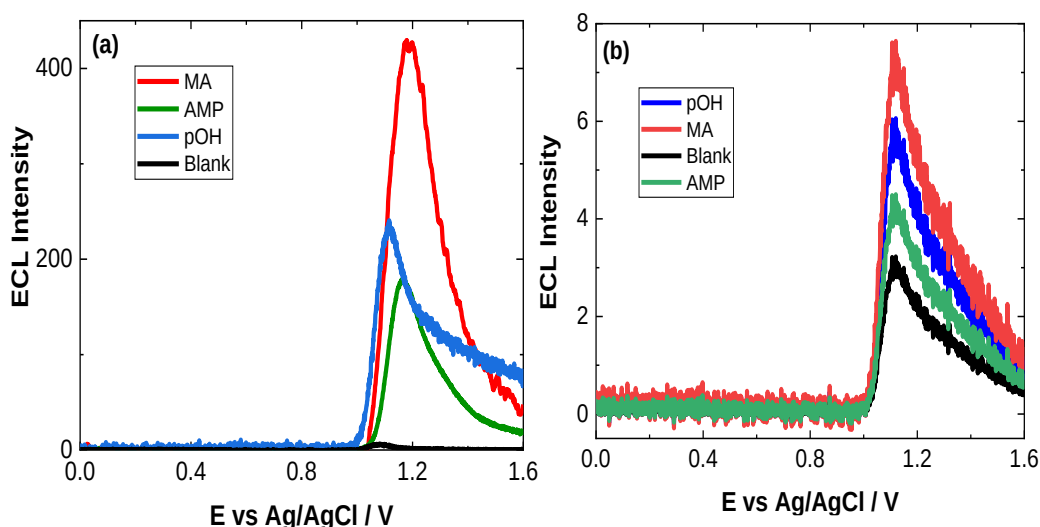


Figure 4.13. (a) ECL of 200 μ M MA (red), 200 μ M AMP (green), 200 μ M pOH-MA (blue) and a blank (black) in urine after a 1:4 dilution (v/v) with 0.1 M PBS (pH = 9.0) scanned over the potential range $0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V. (b) ECL of 0.5 μ M MA (red), 2 μ M AMP (green) and 4 μ M pOH-MA in urine after a 1:4 dilution (v/v) with 0.1 M PBS (pH = 9.0) scanned over the potential range $0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

In the literature, some previously reported voltammetric sensors with no modification to the working electrode have approximately LoD of 10^{-6} and generally working range is from 10^{-6} to 10^{-3} .⁴³⁻⁴⁶ Some voltammetric sensors with modified working electrode show lower LoDs.^{47,48} A screen-printed electrode was modified with gold nanoparticles and that system has LoD of 6.0 nM.⁴⁷ However, these systems are optimised for a single matrix and cannot be used in the same format for other biological matrices. Therefore, although the LoD for this study does need to be improved

further, its applicability to use in portable devices where the sample matrix may not be known prior to attending a crime scene or arriving in an emergency room is a significant advantage. In addition, the ability to detect not just one ATS but AMP, MA and *p*OH-MA is also advantageous for portable screening. Future work examining the potential of a ratiometric approach¹⁹ to improve selectivity and investigation of alternative supports or the inclusion of nanoparticles to enhance the ECL response,⁴⁹⁻⁵² thereby increasing the sensitivity are underway.

Table 4.2. % recovery of different concentrations of MA, AMP and p-OH-MA in body fluids

[ATS] μ M	Neat % recovery	Saliva % recovery (± 4.68)	Human Serum % recovery (± 6.22)	Urine % recovery (± 4.45)
[MA] 150		76.73	76.70	88.90
[MA] 200	41.31 (Urine) 3.18 (Serum)	91.27	89.73	89.29
[MA] 250		87.31	91.45	87.71
[AMP] 150		85.57	91.56	88.49
[AMP] 200	22.29 (Urine) 11.43 (Serum)	90.41	94.63	91.72
[AMP] 250		94.22	92.88	96.12
[pOH-MA] 150		92.77	86.44	94.48
[pOH-MA] 200	40.62 (Urine) 5.80 (Serum)	97.55	81.73	92.54
[pOH-MA] 250		89.6	88.57	91.24

When the results from HPLC, MA in human serum was detected by ECL method and a simple liquid-liquid extraction was performed to decrease the background signal from the interference. LoD was found as 0.5 μ M (signal to noise, 1:3) and % recovery ratio was ~94%.³⁸ Also, HPLC-MS method was studied for detection of some of amphetamine-type stimulants in human urine. LoD for MA was 1 nM and linear working range was 36.1 nM to 0.38 mM. In this method, there was a solid phase extraction step to

decrease the effect of interferences in human urine.²¹ GS-FID (flame ionization detector) was used to detect AMP in urine. LoD for AMP was 2 nM and linear working range was 7.4 nM to 14.8 μ M.⁴⁰ *p*OH-MA in urine was analysed by HPLC and % recovery was seen as % 86.5 and linear working range was 27.8 μ M to 418 μ M and LoD was 5.2 μ M.⁴¹ Given that the studies in the literature, LoDs for MA, AMP and *p*OH-MA by our ECL system are low enough to meet to the requirements in real urine samples without the necessity of an extraction phase. This work illustrates a proof of concept for the screening of ATS in biological matrices, current work is underway to further improve both the sensitivities as well as approaches to provide more selectivity, although in initial triage / screening this is not needed.

As evident, natural amino acids from the biological samples do produce a signal (see Figures 4.9, 4.12 and 4.13). To further assess the specificity of this approach, an interferent study was performed with other compounds including cocaine and diamorphine as well as nicotine. These interferences were assessed against the response of MA and can be found in Figure 4.14. As shown both cocaine and diamorphine at similar concentrations produce comparable ECL responses. Previous aptamer-based sensors also saw responses from cocaine.⁵³ Other non-illicit substances were also assessed, namely nicotine and paracetamol. These do produce responses in the same range as the blank or neat biological samples themselves. This does represent a limitation of the current of the proposed approach, this is outweighed by the potential screening ability of this portable ECL approach and the rapid results that can be obtained. Despite these interference

effects, we are confident in the identification of the presence of illicit drugs including ATS from this screening approach.

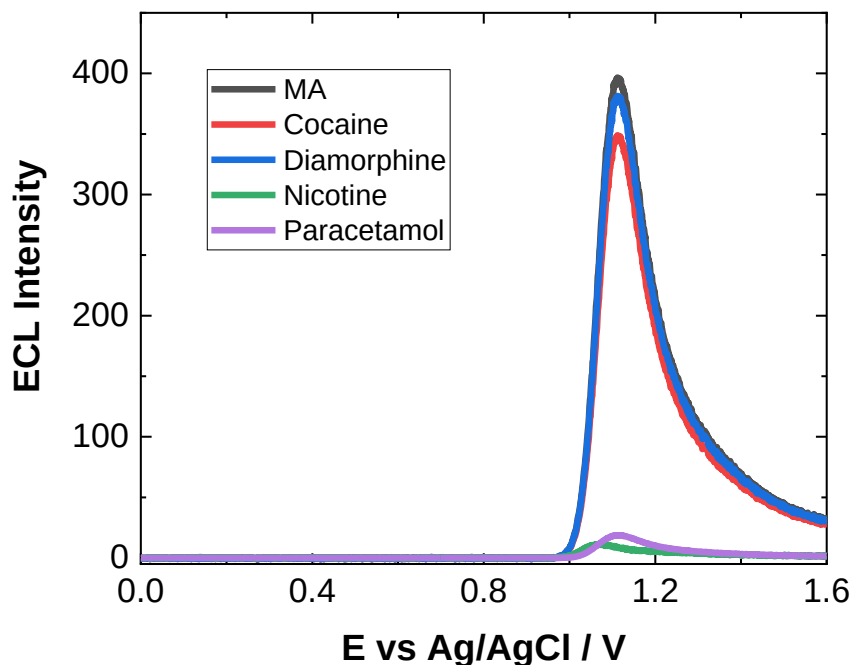


Figure 4.14. (a) ECL of 150 μM MA (black), 150 μM cocaine (red), 150 μM diamorphine (blue), 150 μM nicotine (green) and 150 μM paracetamol (purple) in 0.1 M PBS ($\text{pH} = 9.0$) scanned over the potential range $0 \leq E \leq 1.6$ V vs Ag/AgCl at a scan rate of 100 mVs^{-1} . PMT was biased at 650 V.

4.3. Conclusion

In this work we demonstrated the ability of a simple nafion- $[\text{Ru}(\text{bpy})_3]^{2+}$ film modified electrode to detect MA alongside two of its metabolites within a variety of biological matrices. Unfortunately, the current approach does not provide the ability to discriminate between the different ATS present, although it is possible to confidently determine that at least one of these ATS is present. Determining the presence of an ATS is still extremely

useful for screening or triage purposes for portable detection. Future work into gaining this ability to discriminate between structurally similar complexes is currently underway. This approach highlights the ability of this ECL based sensor to be applied to non-ideal biological matrices with little to no sample preparation and no extraction required, thereby being ideally suited for implementation within portable and on-site screening. This showcases the potential of ECL sensors for portable detection.

**Chapter 5- ECL efficiency of Iridium and Osmium
complexes to detect methamphetamine and its
main metabolites**

Abstract

The majority of ECL sensors rely on the generation of ECL from reactions with ruthenium, this has limited the application of ECL sensors to the detection of one analyte. This presents challenges for selectivity and multiplexed detection particularly for analysis within biological matrices. This is due to the high number of natural amines, reactive oxygen species, as well as other common small molecules present within biological matrices which all react with $[\text{Ru}(\text{bpy})_3]^{2+}$ to produce an ECL signal. This means that control samples can often have ECL signals, limiting the sensitivity of the sensor. The aim of the work is to detect and distinguish MA, AMP, and p-OH-MA by using different Ir (III) complexes and osmium complexes on the modified electrode system. ECL of two iridium (III) complexes ($[\text{ppy}_2\text{Ir}(\text{bpy})]^+$, $(\text{pq})\text{Ir}(\text{acac})$ (ppy = 2-phenylpyridine anion, pq = 2-phenylquinoline anion, pby = 2,2'-bipyridine and acac = acetylacetonate anion) and three osmium complexes ($[\text{Os}(\text{dmpb})_2(\text{diarsp})]^{2+}$, $[\text{Os}(\text{bthp})_2(\text{dmpe})]^{2+}$, $[\text{Os}(\text{diars})_2(\text{bthp})]^{2+}$ (diars : 1,2-bis(dimethylarseno)benzene, dmpb : 1,2-bis(dimethylphosphino)ethane, bthp : 4,7-diphenyl-1,10-phenanthroline, dmpe : 1,2-bis(dimethylphosphino)ethane) in nafion film was investigated at a GC electrode. The experimental conditions, such as potential, pH, concentrations were investigated. However, the modified electrode systems do not show ECL signal against MA and its metabolites under the experiment conditions.

5. Introduction

Since the 1970s, when first electrogenerated chemiluminescence reaction (ECL) was observed between $[\text{Ru}(\text{bpy})_3]^{3+}$ ("bpy" is 2,2-bipyridine) and tripropylamine (TPA)³¹, ECL methods are used in a wide range of analytical applications like immuno assays, detection of infectious diseases, tests for cancer, cardiac, diabetes, as well as environmental monitoring including analysing food and water samples.¹⁰⁹⁻¹¹¹ ECL has absence of a background optical signal and the ability for controlling reaction kinetics by adjusting applied potential, capability with solution-phase and thin film modifications.

The majority of ECL sensors rely on the generation of ECL from reactions with ruthenium, this has limited the application of ECL sensors to the detection of one analyte and also presented challenges for selectivity and multiplexed detection particularly for analysis within biological matrices. This is due to the high number of natural amines, reactive oxygen species, as well as other common small molecules present within biological matrices which all react with $[\text{Ru}(\text{bpy})_3]^{2+}$ to produce an ECL signal. This means that control samples can often have ECL signals, limiting the sensitivity of the sensor. In addition, if discrimination between two structurally similar analytes is needed and both react with ruthenium, this cannot be achieved thereby limiting the selectivity of the sensor design. To illustrate this challenges, cyclometalated iridium complexes ($[\text{ppy}2\text{Ir}(\text{bpy})]^+$, $[\text{ppy}2\text{Ir}(\text{phen})]^+$, $(\text{pq})2\text{Ir}(\text{acac})$, and $(\text{pq})2\text{Ir}(\text{tmd})$) were studied and the ECL results showed that $(\text{pq})2\text{Ir}(\text{acac})$ and $(\text{pq})2\text{Ir}(\text{tmd})$ gave 77 and 49 times higher ECL signal compared to $[\text{Ru}(\text{bpy})_3]^{2+}$ / TprA system.¹²⁰ Extremely high ECL has been obtained because Ir(III) complex

is a strong emitter due to a neutral 2-phenylquinoline group, TPA⁺⁺ can be generated effectively and Ir(III) complex accepts electrons more efficiently from TPA*. (see Equation 5.1-5.5)



An ECL system using Ir(III) complexes was fabricated.¹⁰⁵ F(Ir)pic (bis(3,5-difluoro-2-(2-pyridyl)phenyl)-(2-carboxypyridyl))- iridium III) and (btp)2Ir(acac) (bis[2,(2'-benzothienyl)-pyridinato-N,C3](acetylacetonate)Ir(III)) was investigated in acetonitrile (MeCN), mixed MeCN/H₂O (50:50, v/v), and aqueous solutions using the oxidative reductive coreactant tri-n-propylamine. The ECL emission spectrum of F(Ir)pic can be distinguished from [Ru(bpy)₃]²⁺ when both are present in the same solution. ECL of F(Ir)pic showed higher intensity when compared with [Ru(bpy)₃]²⁺ and ECL peak of F(Ir)pic was distinguished from that of [Ru(bpy)₃]²⁺.¹⁰⁵

ECL reaction pathway is identified for $[\text{Ru}(\text{bpy})_3]^{2+}$ and TPrA co-reactant (see chapter 1.4). However, novel electrochemiluminophores cannot follow the same pathway always and that is an important point for intended applications. For example, in an ECL system where metalo-luminophores (Ir (III) complexes) are immobilized on an electrode surface.¹¹⁶ There, ECL is due to diffusion of oxidised TPrA to electrochemiluminophores. It was seen that the reaction (5.6) and (5.8) are not feasible for most of iridium complexes.¹¹⁶ Another iridium complex work showed that the reactions from (5.1) to (5.5) became very important to see ECL intensity.^{109,119}

In recent years, new ECL systems using iridium and osmium complexes were published.^{105,108} These iridium and osmium complexes showed high and reproducible ECL signal in the presence of TPA. In the literature, there are studies to develop novel monomeric or multicentre metal complexes.^{98,99} ECL luminophores are created by adding functional groups to one or two pyridine rings of a single ligand. Those modifications cause of enhanced electrochemical and photophysical properties of the luminophore.¹¹² Excited-state location within the complexes, luminescence lifetime, ground and excited state potentials can be adjusted for special purpose and all these features directly affect the performance of an ECL system.^{98,99} For example, a newly designed luminophore, can show longer excited-state lifetime and this makes the ECL system more sensitive because longer lifetimes cause brighter ECL response. Some examples from the literature is seen in Figure 5.1.

Researchers published some dimeric metal complexes because this type of luminophores generally produce two or three-fold more intense emission in aqueous and nonaqueous medium.³¹ However, there is an

upper limit of intensity when more complex luminophores are used due to problem of diffusion rate and heterogeneous electron transfer.³¹

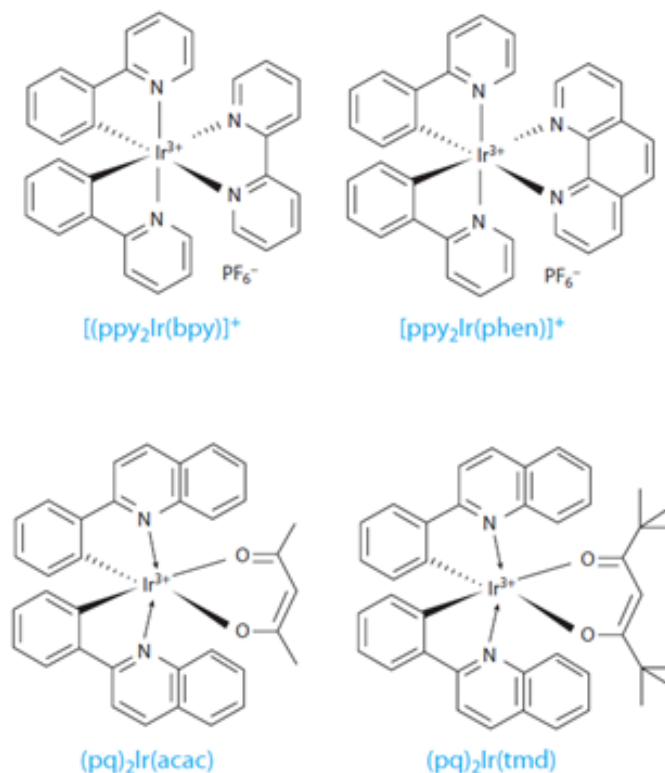


Figure 5.1 Some iridium complexes used in electrochemiluminescence studies.^{105,106}

Cyclometallated iridium (III) complexes have been used as a new generation of ECL labels.^{113,115} When compared with [Ru(bpy)₃]⁺², Iridium complexes show enhanced analytical performance.¹¹⁶ Those electrochemical properties of Ir (III) bipyridine complexes made researchers began to examine new type of cyclometalated iridium complexes which have high ECL efficiencies, a wide range of emission

wavelengths and high selectivity¹⁰⁶ and multi-colour detection systems (multiplexed systems).¹⁰⁸

In the literature, the structure of ruthenium, iridium and osmium polypyridine type complexes are generally used (Figure 5.2). These structures have almost ideal reversible voltammetry and effective photophysical properties.⁴⁰

Osmium complexes are another important ECL luminophores. Generally, they have shorter excited-state lifetimes and weaker emission when compared with iridium complexes. That is due to the fact that osmium complexes have larger spin-orbit coupling and that is a limitation for ECL production.¹⁰⁷ However, osmium complexes may have advantage because they are more photostable than ruthenium complexes and are oxidised at less anodic potentials. For example, $[\text{Os}(\text{phen})_2(\text{dppene})]^{2+}$ was used as luminophore with TPA in aqueous medium. ECL efficiency was recorded as two-fold higher than that of $[\text{Ru}(\text{bpy})_3]^{2+}$.¹⁰⁸

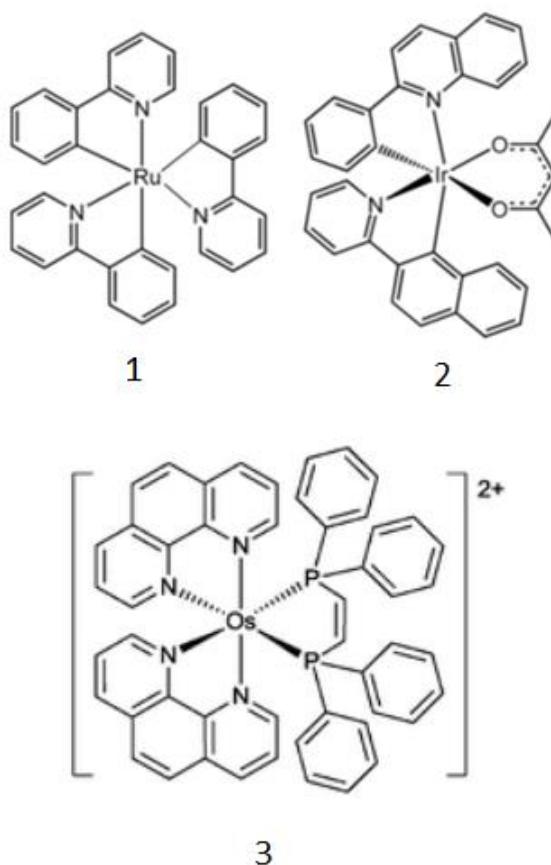


Figure 5.2 Examples of ECL luminophores are 1- $\text{Ru}(\text{bpy})_3^{2+}$, 2- $(\text{pq})_2\text{Ir}(\text{acac})$ and 3- $[\text{Os}(\text{phen})_2(\text{dppene})]^{2+}$

Development of portable and highly sensitive ECL devices such as biomedical and environmental research have more attention these days. Due to high sensitivity and selectivity of ECL systems, more applications in different fields come up.³¹ Also, sensitivity and selectivity of ECL systems can be enhanced by advanced materials like interfacial films, nanoparticles, and new complexes.

The aim of the project is to detect and distinguish MA, AMP, and p-OH-MA by using different Ir (III) complexes and osmium complexes on the modified electrode system. A mixed metal system such as ruthenium,

iridium and osmium complex can be created and the individual luminophores work for the individual ATS for multi-analyte sensing. That modified electrode system can be used for body fluids and the concentration of MA, AMP and p-OH MA can be calculated separately by using only one electrode system.

5.1. Experimental

5.1.1. Materials

$[\text{Ru}(\text{bpy})_3]^{2+}$ (Tris (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate), $\text{Ir}(\text{ppy})_3$ (Tris2-phenylpyridinato- C^2N iridium (III)), $\text{Ir}(\text{pq})_2(\text{acac})$ ((2,4-Pentanedionat) bis2-(2-quinoliny) phenyliridium (III)) (see Figure 5.3) Nafion 117 (per-flourinated ionexchange resin, 5% (w/v) solution in a mixture of alcohols) , acetonitrile was purchased from Sigma-Aldrich. Phosphate buffer (PBS, 0.1 mol/L) was used to control the pH of aqueous solution (+) methamphetamine hydrochloride and DL-amphetamine were purchased from Sigma-Aldrich. 3 of Osmium complexes synthesized by Australian working group were used (see Figure 5.4). All other reagents were of analytical reagent grade and ultra-pure water (Milli-Q plus, Millipore Inc.) was used throughout the experiments.

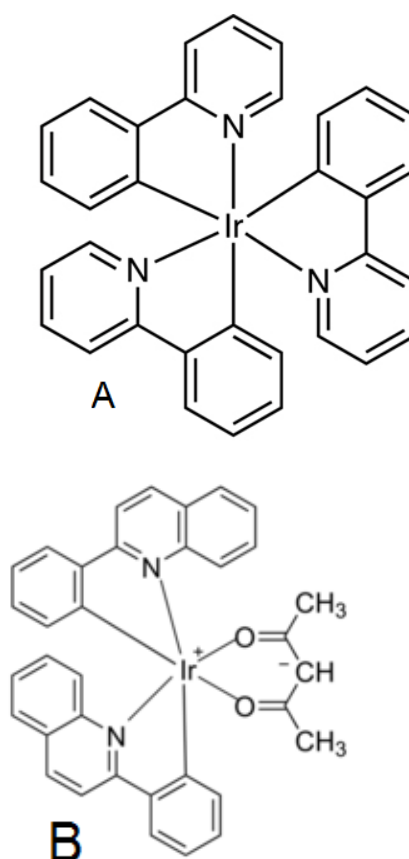


Figure 5.3 Iridium complexes used in the study. **A** is $[ppy_2Ir(bpy)]^+$ and **B** is $(pq)Ir(acac)$. (*ppy* = 2-phenylpyridine anion, *pq* = 2- phenylquinoline anion, *bpy* = 2,2'-bipyridine and *acac* = acetylacetonate anion).

A three-electrode configuration was used. A glassy carbon electrode (GC) was coated with Iridium and osmium complexes / Nafion film as working electrode. A platinum wire electrode and Ag/AgCl (saturated KCl) were used as counter electrode and reference electrode, respectively. All the potentials were measured and reported according to this reference electrode. The ECL experiments were carried out by a chemiluminescence analyser (CH instrument model 602E connected to a Hamamatsu H6780-20 PMT). The ECL cell was placed directly in front of the photomultiplier tube (PMT) and enclosed in a light -tight box.

Before modification of GC electrode (d:3 mm), it was polished with 1, 0.3 and 0.05 μm aluminum solution on a polishing cloth, respectively and sonicated in water and washed with water. Then, it was dried.

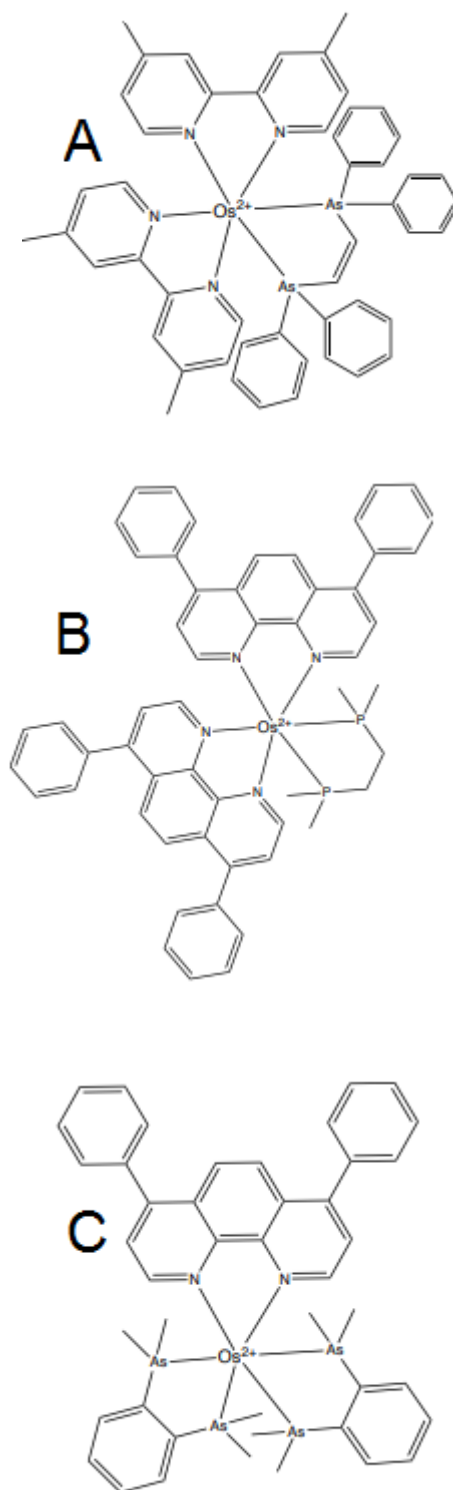


Figure 5.4 Osmium complexes used in the study. A: $[\text{Os}(\text{dmpb})_2(\text{diarsp})]^{2+}$, B: $[\text{Os}(\text{bthp})_2(\text{dmpe})]^{2+}$, C: $[\text{Os}(\text{diars})_2(\text{bthp})]^{2+}$ (diars: 1,2-bis(dimethylarseno)benzene, dmpb: 1,2-bis(dimethylphosphino)ethane, bthp: 4,7-diphenyl-1,10-phenanthroline, dmpe: 1,2-bis(dimethylphosphino)ethane)

5.2. Results and Discussion

5.2.1. Detection of ATS via ECL

The approach utilised within this study was based on the interaction of ATS with Iridium and osmium complexes, which are mentioned in the experimental section (see Figure 5.3 and Figure 5.4). The aim was to see ECL signal upon interaction with iridium and osmium complexes through the oxidative-reductive pathway. MA, AMP, and p-OH-MA were studied as analyte. By using the interaction between different ATS and different metal complexes, we are aiming to distinguish each ATS in samples and we can have more specific ECL system for each ATS.

5.2.2. $\text{Ir}(\text{pq})_2(\text{acac})$ / nafion modified GC electrode

The $\text{Ir}(\text{pq})_2(\text{acac})$ /nafion film modified glassy carbon electrode was fabricated by the following procedure: A 1 mM $\text{Ir}(\text{pq})_2(\text{acac})$ solution was prepared in 50:50 EtOH:H₂O. 5 % w/v nafion was diluted to a concentration of 0.4 % w/v with 50:50 EtOH:H₂O. The final film concentration of 0.5 mM $\text{Ir}(\text{pq})_2(\text{acac})$ and 0.2 % nafion was prepared by mixing the above solutions in a 1:1 (v/v) ratio. Once prepared the film was stored at 4 °C. The carbon working electrode modified with the film and left to air dry for 2 hours under darkness.

The modified sensor was immersed into 0.1 M PBS in the presence of MA, AMP, and p-OH MA. The ECL measurement was performed at a potential range from 0.0 V to 1.6 V vs reference electrode (Ag/AgCl saturated with KCl) at scan rate of 100 mVs⁻¹ with a conventional three-electrode system. The GC electrode modified with $[\text{ppy}_2\text{Ir}(\text{bpy})]^+$ / nafion was used as working electrode.

Experimental parameters and conditions directly effect ECL intensity. Some studies reported experimental intensities for new complexes under chemical conditions where the reference luminophore ($[\text{Ru}(\text{bpy})_3]^{2+}$) has disadvantages.^{106, 117, 118} In this context, a range of pH from 7 to 11 was studied to check pH effect on ECL intensity. Background signals of the modified electrode were shown in Figure 5.5.

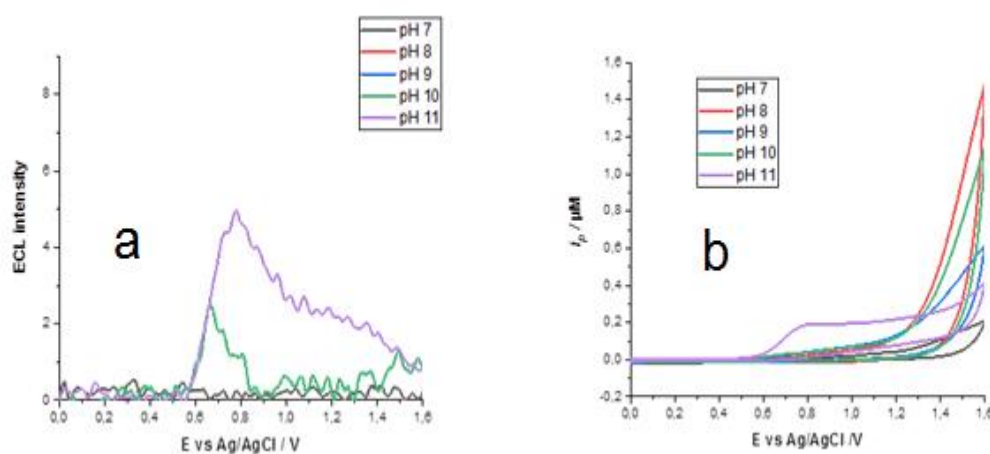


Figure 5.5 (a) is ECL signals of blanks of the modified GC electrode with $(\text{pq})_2\text{Ir}(\text{acac})$, **(b)** is CVs of blanks of the modified GC electrode with $(\text{pq})_2\text{Ir}(\text{acac})$ at a range of from pH 7 to pH 11 in 0.1 M PBS at a scan rate of 100 mVs^{-1} over the potential range of 0 to 1.6 V vs Ag/AgCl.

MA, AMP and p-OH-MA were studied under the same condition by the GC electrode modified with $(\text{pq})_2\text{Ir}(\text{acac})$ / Nafion. The ECL responses were shown in Figure 5.6.

Based on the results from Figure 5.5 and 5.6, ECL signal of the blank samples and in the presence of $100 \mu\text{M}$ of the ATs are the same. Also, at higher pH values, there is an increasing background ECL signal and that

may be since effect of OH^- at higher pH levels.^{120,121} That effect was also mentioned in the previous chapters (see Chapter 2 and 3). On the other hand, in the CVs of the blank and the ATSS responses at higher pH levels, there are very similar oxidation peak at about 0.7 V. Again, that oxidation peak is most probably due to OH^- groups and $\text{Ir}(\text{pq})_2(\text{acac})$ complex.¹²¹

Figure 5.6 shows that, in the presence of MA and the main metabolites, the system shows very similar ECL intensity as the background signal in the blank. Obviously, the modified electrode system has no meaning ECL signal for MA, AMP, and pOH-MA.

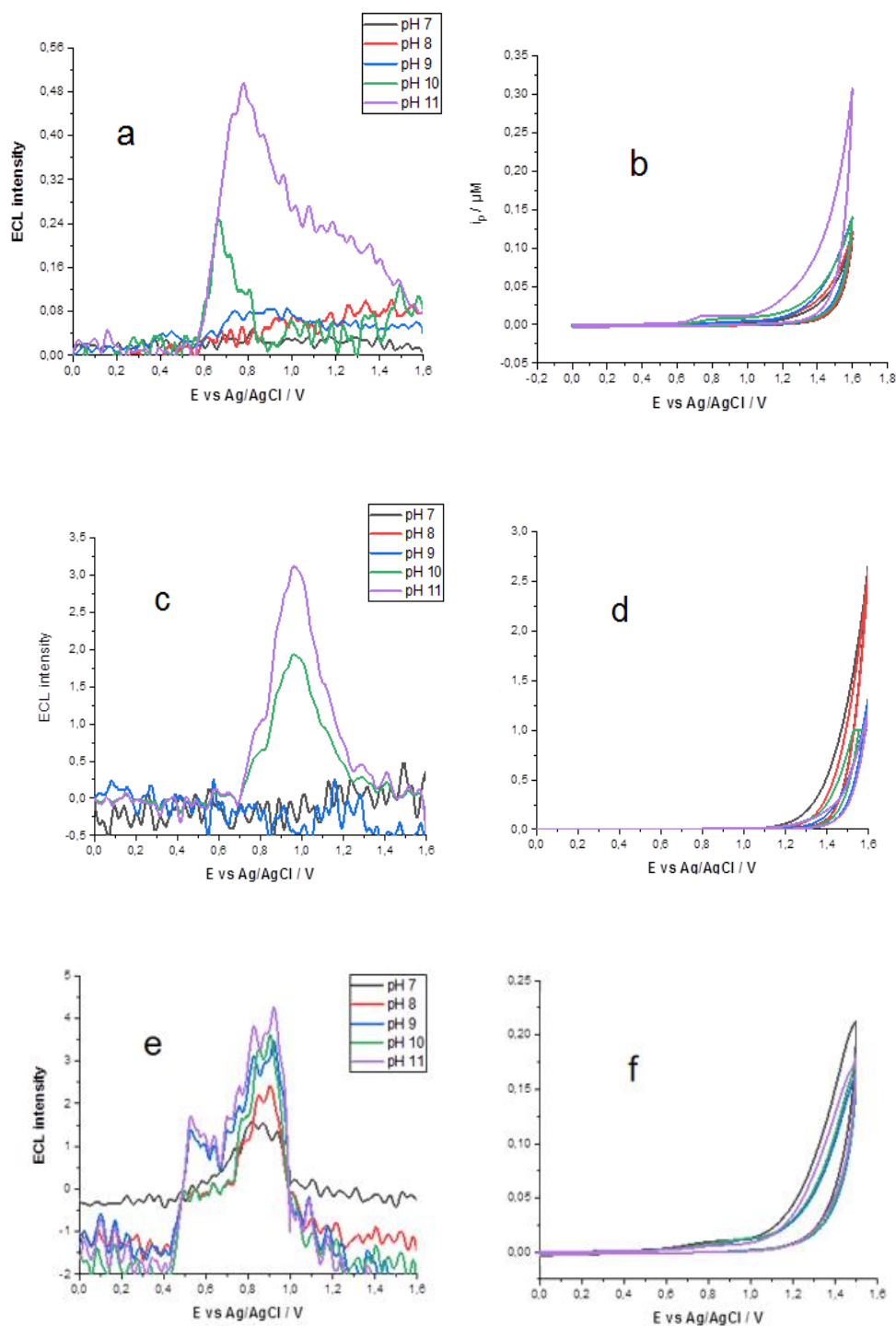


Figure 5.6 (a) ECL responses of 100 μM MA, (b) CVs of 100 μM MA, (c) ECL responses of 100 μM AMP, (d) CVs of 100 μM AMP, (e) ECL responses of 100 μM p-OH, (f) CVs of 100 μM p-OH in a range of pH of 0.1 M PBS (pH 7 to pH 11) at a scan rate of 100 mVs⁻¹ over the potential range of 0 to 1.6 V vs Ag/AgCl.

5.2.3. Ir(ppy)₃ / nafion modified GC electrode

The sensor fabricated was immersed into 0.1 M PBS in the presence of MA, AMP, and p-OH MA. The ECL measurement was performed at a potential range from 0.0 V to 1.6 V at scan rate of 100 mVs⁻¹ with a conventional three-electrode system. Glassy carbon electrode fabricated with Ir(ppy)₃ / nafion was used as working electrode. Background signals of the modified electrode were shown in Figure 5.7.

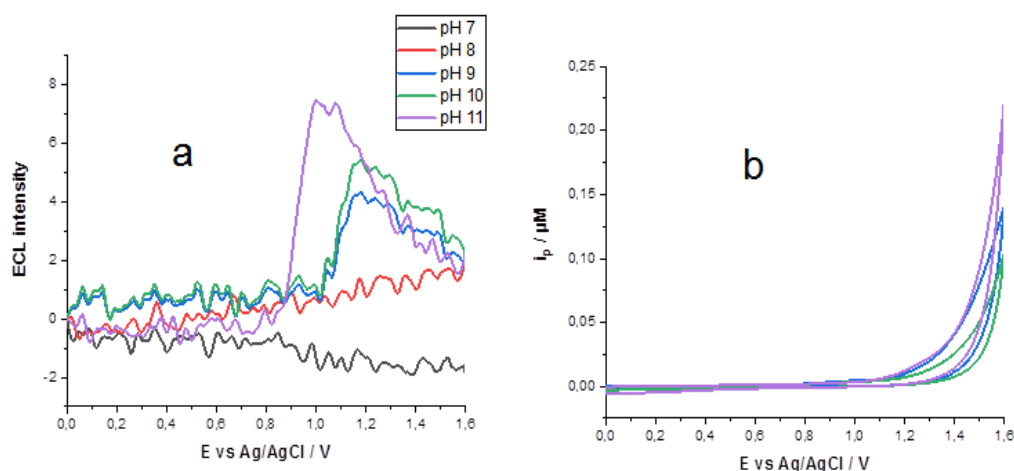


Figure 5.7 (a) is ECL signals of blanks of the modified GC electrode with Ir(ppy)₃, **(b)** is CVs of blanks of the modified GC electrode with Ir(ppy)₃ at a range of from pH 7 to pH 11 in 0.1 M PBS at a scan rate of 100 mVs⁻¹ over the potential range of 0 to 1.6 V vs Ag/AgCl.

MA, AMP and p-OH-MA were studied under the same condition by the GC electrode modified with Ir(ppy)₃ / Nafion.

An increasing background signal was seen in the blank at higher pH levels. Here, that background signal is due to interaction between Ir(ppy)₃

and OH^- ions.^{120,121} The ECL responses in the presence of the ATS were shown in Figure 5.8. Very similar results were observed in Figure 5.8. there is an increasing ECL signal depending the pH level. Based on the results from Figure 5.7 and 5.8, it clearly can be said that ECL signal of the blank samples and in the presence of 100 μM of the ATSS are indistinguishable.

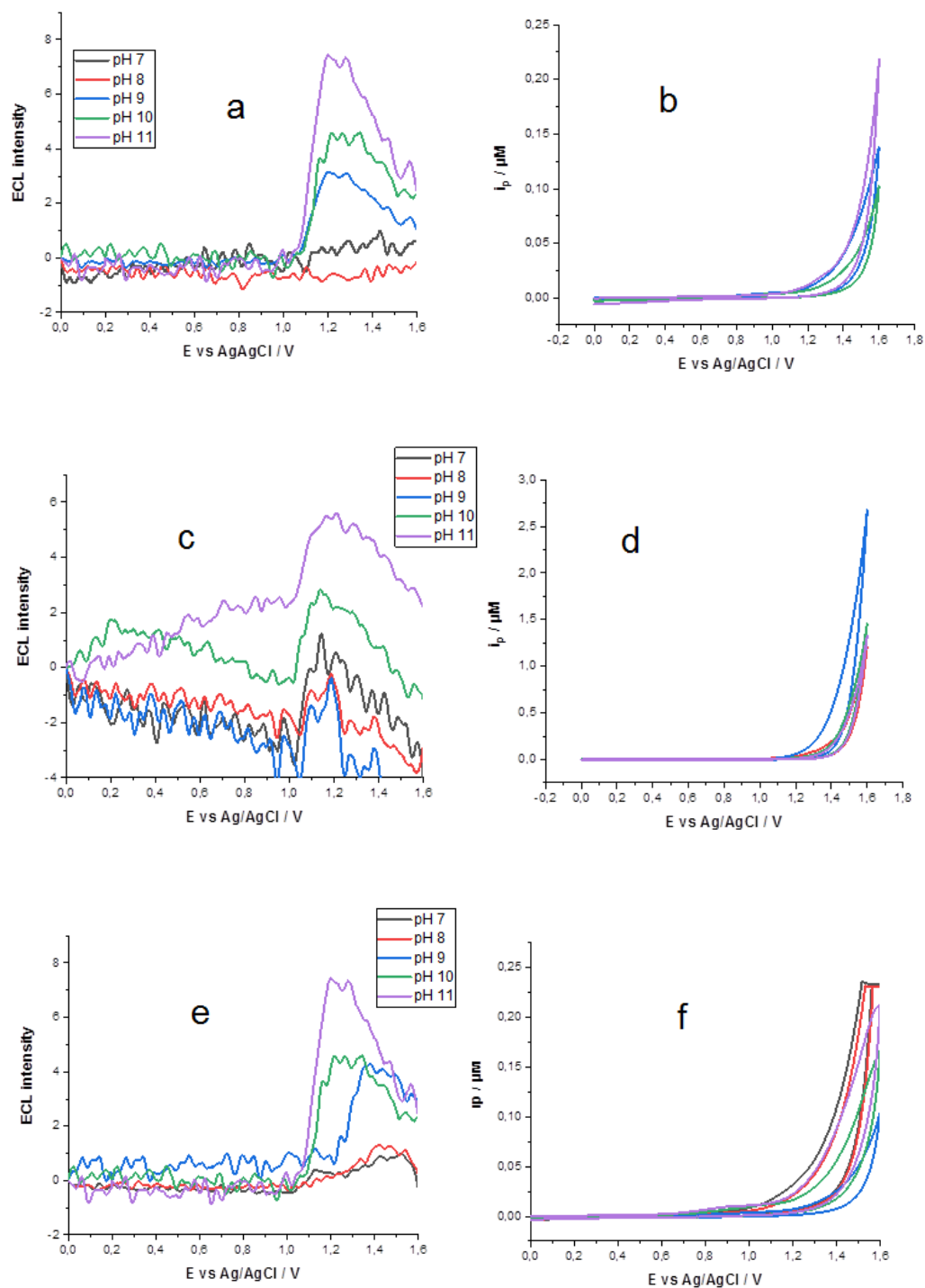


Figure 5.8 (a) ECL responses of 100 μM MA, (b) CVs of 100 μM MA, (c) ECL responses of 100 μM AMP, (d) CVs of 100 μM AMP, (e) ECL responses of 100 μM p-OH, (f) CVs of 100 μM p-OH in a range of pH of 0.1 M PBS (pH 7 to pH 11) at a scan rate of 100 mVs^{-1} over the potential range of 0 to 1.6 V vs Ag/AgCl.

5.2.4. [Os(dmpb)₂(diarsp)]²⁺ / nafion modified GC electrode

The [Os(dmpb)₂(diarsp)]²⁺ /nafion film modified glassy carbon electrode was fabricated by the following procedure: A 1 mM osmium complex solution was prepared in 50:50 ACN: H₂O. 5 % w/v nafion was diluted to a concentration of 0.4 % w/v with 50:50 ACN: H₂O. The final film concentration of 0.5 mM [Os(dmpb)₂(diarsp)]²⁺ and 0.2 % nafion was prepared by mixing the above solutions in a 1:1 (v/v) ratio. Once prepared the film was stored at 4 °C. The carbon working electrode modified with the film and left to air dry for 2 hours under darkness.

The osmium complexes were immersed into 0.1 M PBS but PBS showed very intense ECL signal in blanks and NaCl was used as electrolyte to get rid of unwanted ECL peak. So, the modified sensor was immersed into 1 M NaCl in the presence of MA, AMP, and p-OH MA. The osmium complexes are all made up to a 1 mM concentration in 50:50 ACN: H₂O solvent system, this is the solvent system to make them up in to get them to dissolve, any more water causes of crashing out them.

The ECL measurement was performed at a potential range from 0.0 V to 1.6 V vs Ag/AgCl (saturated with KCl) at scan rate of 100 mVs⁻¹ with a conventional three-electrode system. Glassy carbon electrode fabricated with osmium complex / nafion was used as working electrode. Background signals were shown in Figure 5.9.

In the CVs of the blank at higher pH levels, there is an oxidation peak. Oxidation and reduction peaks are most probably due to nonhomogeneous electron transfer between the subgroups of osmium complex at higher pH. That means subgroups lose electron at higher pH levels and that causes

of an oxidation peak in the CVs. Similar results were seen for the other metal complexes in the study.

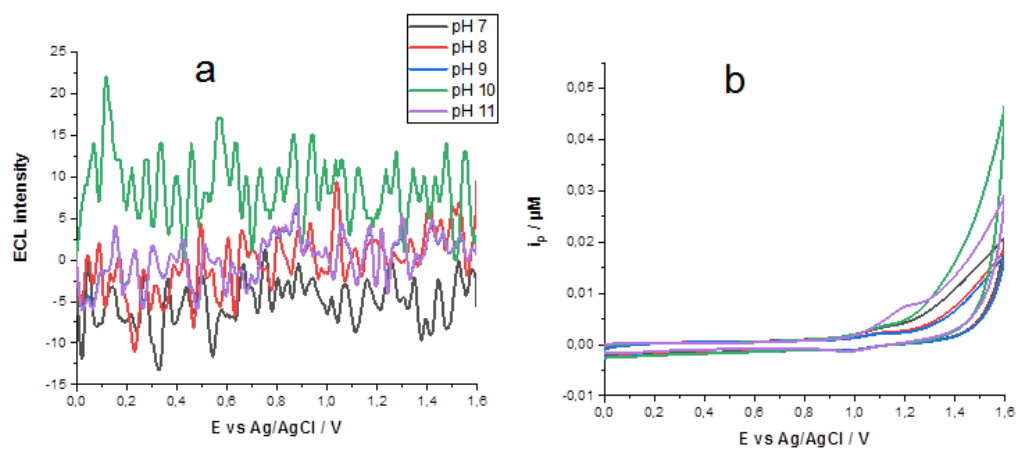


Figure 5.9 (a) is ECL signals of blanks of the modified GC electrode with $[\text{Os}(\text{dmpb})_2(\text{diarsp})]^{2+}$, (b) is CVs of blanks of the modified GC electrode with $[\text{Os}(\text{dmpb})_2(\text{diarsp})]^{2+}$ at a range of from pH 7 to pH 11 in 1 M NaCl at a scan rate of 100 mVs⁻¹ over the potential range of 0 to 1.6 V vs Ag/AgCl.

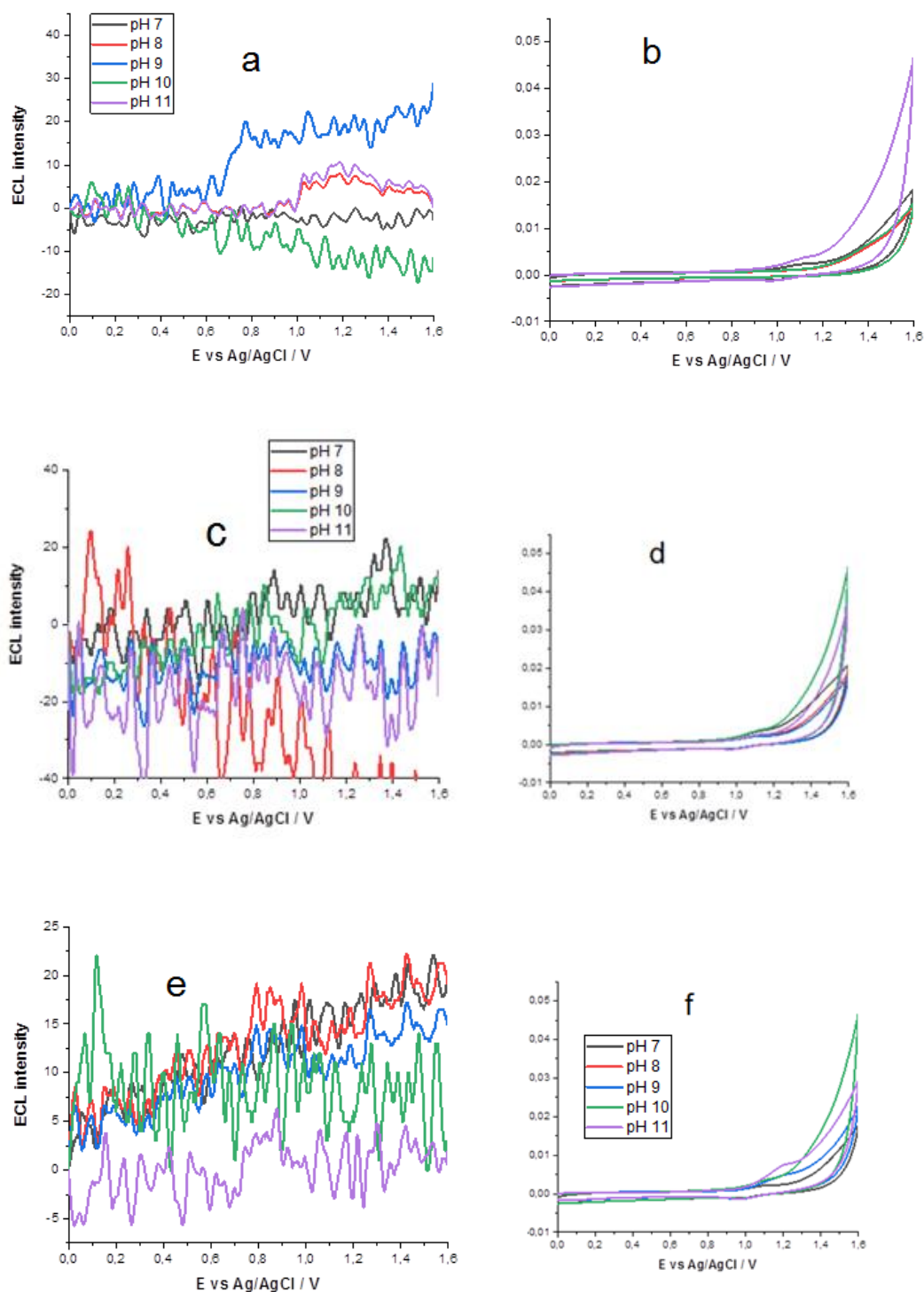


Figure 5.10 (a) ECL responses of 100 μM MA, (b) CVs of 100 μM MA, (c) ECL responses of 100 μM AMP, (d) CVs of 100 μM AMP, (e) ECL responses of 100 μM p-OH⁻, (f) CVs of 100 μM p-OH⁻ in a range of pH 7 to pH 11 in 1 M NaCl at a scan rate of 100 mVs⁻¹ over the potential range of 0 to 1.6 V vs Ag/AgCl.

5.2.5. $[\text{Os}(\text{bthp})_2(\text{dmpe})]^{2+}$ / nafion modified GC electrode

The $[\text{Os}(\text{bthp})_2(\text{dmpe})]^{2+}$ /nafion film modified glassy carbon electrode was fabricated by the following procedure: A 1 mM osmium complex solution was prepared in 50:50 ACN: H₂O. 5 % w/v nafion was diluted to a concentration of 0.4 % w/v with 50:50 ACN: H₂O. The final film concentration of 0.5 mM $[\text{Os}(\text{bthp})_2(\text{dmpe})]^{2+}$ and 0.2 % nafion was prepared by mixing the above solutions in a 1:1 (v/v) ratio. Once prepared the film was stored at 4 °C. The carbon working electrode modified with the film and left to air dry for 2 hours under darkness.

The ECL measurement was performed at a potential range from 0.0 V to 1.6 V vs Ag/AgCl (saturated with KCl) at scan rate of 0.1 Vs⁻¹ with a conventional three-electrode system. Glassy carbon electrode fabricated with osmium complex / nafion was used as working electrode. Background signals were shown in Figure 5.11. In the CVs of the blank at higher pH levels, there is an oxidation and reduction peak. That oxidation peak is most probably due to nonhomogeneous electron transfer between the subgroups of iridium complex at higher pH. That means subgroups lose electron at higher pH levels and that causes of an oxidation peak in the CVs. Similar results were seen for the other metal complexes in the study.

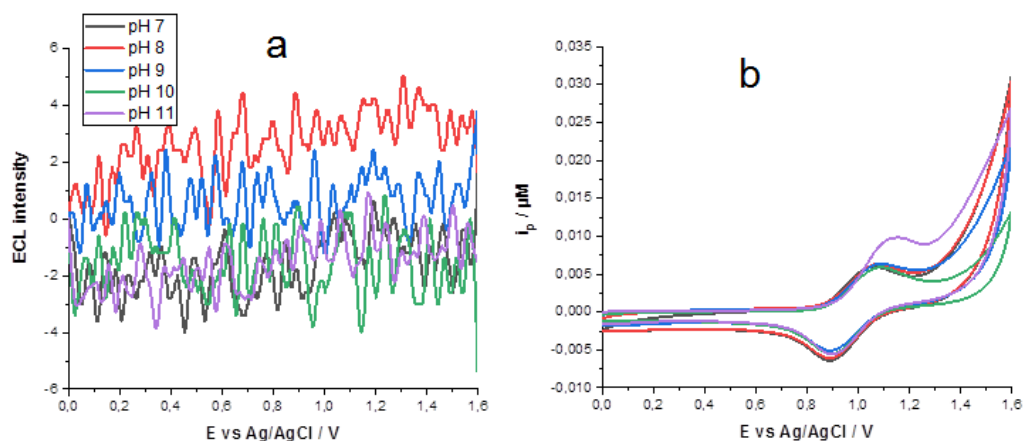


Figure 5.11 (a) is ECL signals of blanks of the modified GC electrode with $[\text{Os}(\text{bthp})_2(\text{dmpe})]^{2+}$, (b) is CVs of blanks of the modified GC electrode with $[\text{Os}(\text{bthp})_2(\text{dmpe})]^{2+}$ at a range of from pH 7 to pH 11 in 1 M NaCl at a scan rate of 100 mVs⁻¹ over the potential range of 0 to 1.6 V vs Ag/AgCl.

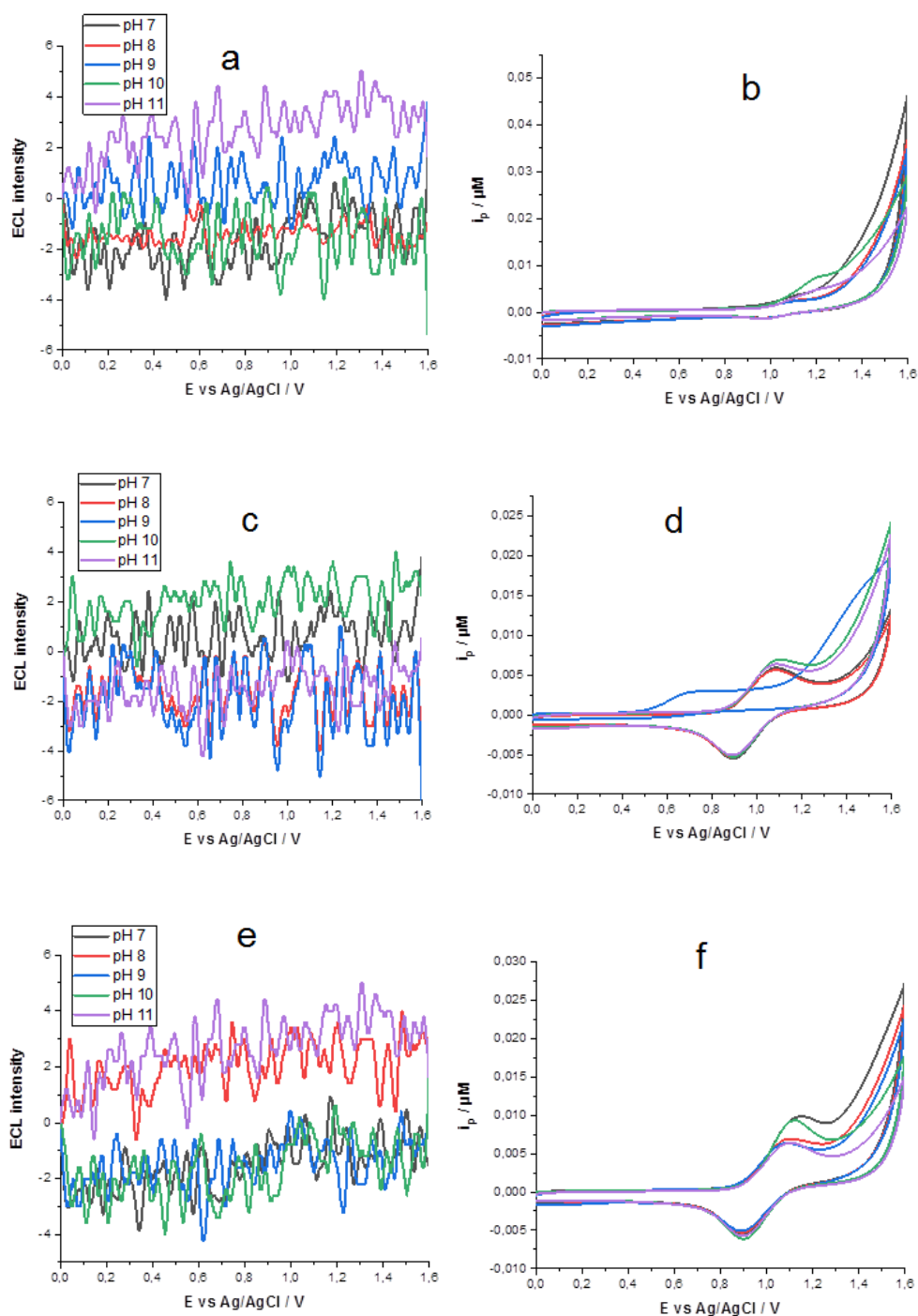


Figure 5.12 (a) ECL responses of 100 μM MA, (b) CVs of 100 μM MA, (c) ECL responses of 100 μM AMP, (d) CVs of 100 μM AMP, (e) ECL responses of 100 μM p-OH⁻, (f) CVs of 100 μM p-OH⁻ in a range of pH 7 to pH 11 in 1 M NaCl at a scan rate of 100 mVs⁻¹ over the potential range of 0 to 1.6 V vs Ag/AgCl.

5.2.6. $[\text{Os}(\text{diars})_2(\text{bthp})]^{2+}$ / nafion modified GC electrode

The $[\text{Os}(\text{diars})_2(\text{bthp})]^{2+}$ /nafion film modified glassy carbon electrode was fabricated by the following procedure: A 1 mM $[\text{Os}(\text{diars})_2(\text{bthp})]^{2+}$ solution was prepared in 50:50 ACN: H₂O. 5 % w/v nafion was diluted to a concentration of 0.4 % w/v with 50:50 ACN: H₂O. The final film concentration of 0.5 mM $[\text{Os}(\text{diars})_2(\text{bthp})]^{2+}$ and 0.2 % nafion was prepared by mixing the above solutions in a 1:1 (v/v) ratio. Once prepared the film was stored at 4 °C. The carbon working electrode modified with the film and left to air dry for 2 hours under darkness.

The ECL measurement was performed at a potential range from 0.0 V to 1.6 V vs Ag/AgCl (saturated with KCl) at scan rate of 0.1 Vs⁻¹ with a conventional three-electrode system. Glassy carbon electrode fabricated with osmium complex / nafion was used as working electrode. Background signals were shown in Figure 5.13. In this complex, there is no oxidation and reduction peak in the CVs. That means that the osmium complex do not show nonhomogeneous electron transfer.

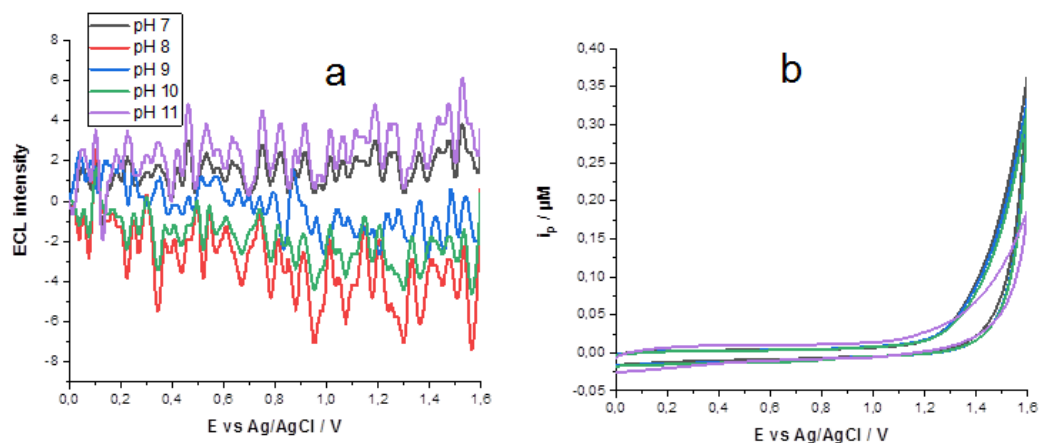


Figure 5.13 (a) is ECL signals of blanks of the modified GC electrode with $[\text{Os}(\text{diars})_2(\text{bthp})]^{2+}$, (b) is CVs of blanks of the modified GC electrode with $[\text{Os}(\text{diars})_2(\text{bthp})]^{2+}$ at a range of from pH 7 to pH 11 in 1 M NaCl at a scan rate of 100 mVs⁻¹ over the potential range of 0 to 1.6 V vs Ag/AgCl.

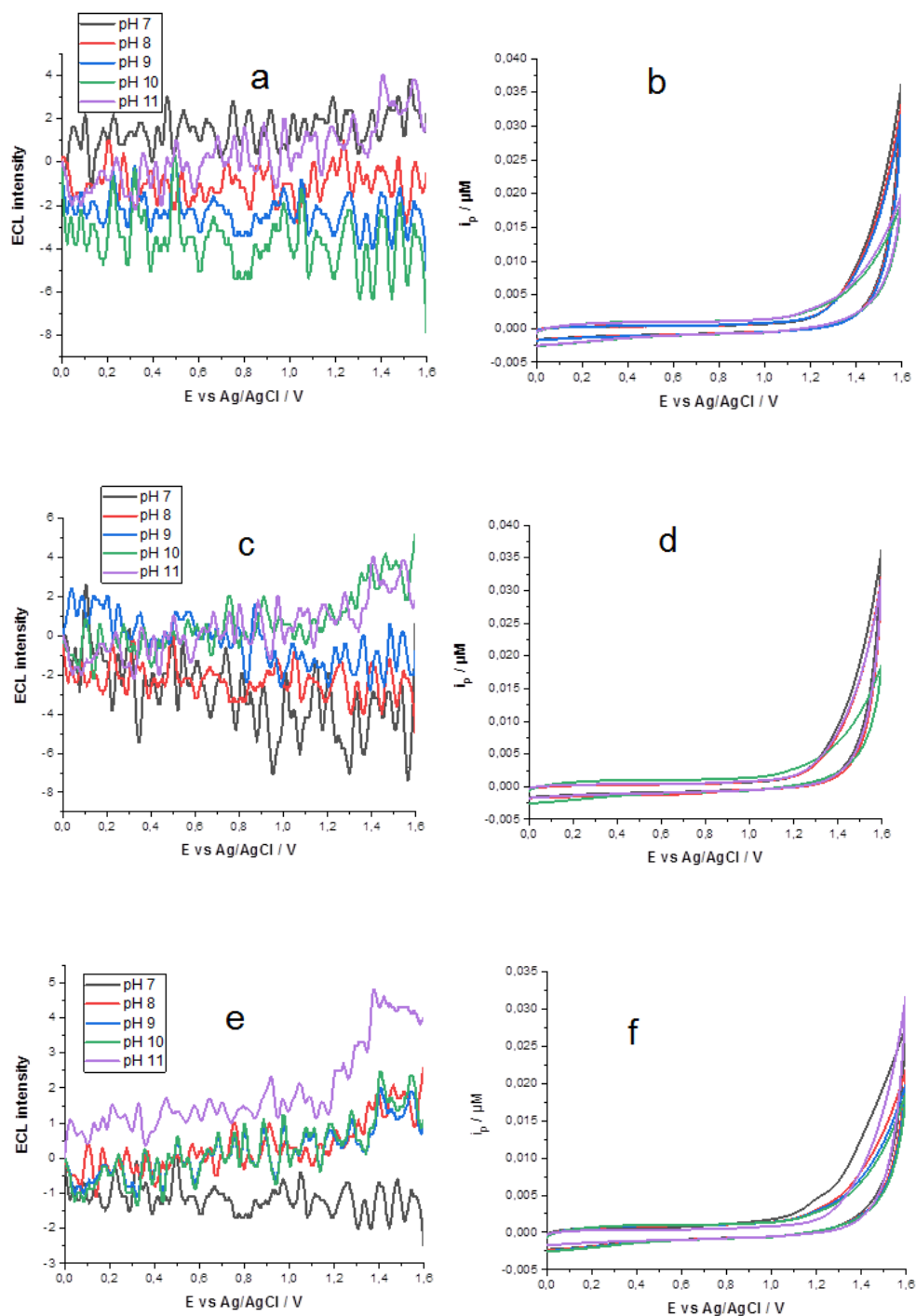


Figure 5.14 (a) ECL responses of 100 μM MA, (b) CVs of 100 μM MA, (c) ECL responses of 100 μM AMP, (d) CVs of 100 μM AMP, (e) ECL responses of 100 μM p-OH⁻, (f) CVs of 100 μM p-OH⁻ in a range of pH 7 to pH 11 in 1 M NaCl at a scan rate of 100 mVs⁻¹ over the potential range of 0 to 1.6 V vs Ag/AgCl.

5.3. Conclusion

The aim was to see ECL signal upon interaction with iridium and osmium complexes through the oxidative-reductive pathway. MA, AMP, and p-OH-MA were studied as analyte. Unfortunately, MA, AMP and p-OH-MA showed no ECL signal against the electrode systems.

To conclude, iridium and osmium complexes were used to distinguish MA and its metabolites in samples. However, the modified electrode systems do not show ECL signal against the analytes. Obviously, these metal complexes can not be utilised for distinguishing MA and its main metabolites within this current system but the metal complexes can be applied for detection of the ATS in different conditions or electrode systems in the future.

6. Conclusion and Future Work

There is an increasing using drugs as recreational item in recent years and the recreational using of drugs has become very common case. The use of recreational drugs has expanded beyond national boundaries. The global estimates of users of amphetamine-type drugs are 0.3 – 1.3 % of the world population ranging 15 to 64 years old. However, the use of illicit drugs in England and Wales is higher than that of lots of European countries and is 8.8 % of the population between 16 to 59 years old. According to a 2020 report published by *the World Drug Report 2020*, England, Scotland and Ireland are major stopping-off points for gangs seeking to supply the European drugs market. Criminal groups have created routes for the sale of methamphetamines (see Figure 1.1). Because of the reasons above, this project focused on synthetic drugs known as ATS.

Traditional methods such as confirmatory methods (HPLC, GC and GC/MS) or some screening methods have a wide range of applications within the scope of forensic science. By using comprehensive chemical and analytical techniques in forensic labs forensic science specialists assess the evidence. In this context, scientists need up-to-date and reliable analytical techniques in order to identify and detect the samples swiftly and with great accuracy. The need for new and more effective systems in forensic field becomes more of an issue because unknown derivatives of illegal drugs, which have more complex structure and are more hazardous for human health, have come to exist over time. Scientists are looking for advanced approaches to solve the current problems. The need for rapid and reliable detection and monitoring in clinical, environmental and

biodefense causes the new ways for development of alternative analytical devices. Electrochemical sensors are strong alternative detection devices and currently electrochemical sensors have a wide range application in food, pathogens, forensic applications, drug response measurement, cancer diagnosis and environmental issues. In this context, we prepared a modified GC electrode with nafion and voltammetry and ECL detection were studied because ECL has a number of important advantages over more conventional chemiluminescence (CL) techniques. Firstly, no external light source is required and therefore instrumentation is simple, background light intensity is minimal and scattered light and interferences from emission by impurities is minimised. Secondly, certain reactants can be regenerated at the electrode, allowing them to undergo ECL reactions more than once thus greatly improving the sensitivity of the technique. This allows the detection of analytes at very low concentrations (picomolar).

According to the CV results (Figure 2.1), the anodic peak potential at 1.05 V results from the oxidation of $[\text{Ru}(\text{bpy})_3]^{2+}$ (see Equation 2.3). If a redox process is reversible then the relationship between the anodic and cathodic peak currents (i_{pa}/i_{pc}) should equate to 1. This therefore indicated that the redox process may have been entirely reversible within this system.

In a diffusion-controlled system, the observed CV response is dependent upon the scan rate of the potential sweep as this determines how rapidly $[\text{Ru}(\text{bpy})_3]^{2+}$ can be oxidised to $[\text{Ru}(\text{bpy})_3]^{3+}$. In this study, these conditions are met $100 \leq \nu \leq 500 \text{ mVs}^{-1}$ and plots of peak current varied linearly with the square root of scan rate over this range, typical cyclic voltammetric scan rate dependency and Randles-Sevcik plots are shown

in Figure 2.3. The system is linearly proportional to scan rate at slow scan rates. This proportionality can often be used to determine the system which can generate $[\text{Ru}(\text{bpy})_3]^{3+}$. This system showed signs of reversibility and the electrode system can be used in 0.1 M PBS media. In this study, aquatic medium was used throughout all the works. Non-aquatic or a mixture of aquatic and non-aquatic solutions can be studied to have more effective and usable analytical and ECL results by compared with the current results.

There are less diffusional issues associated the use of films due to the immobilisation of $[\text{Ru}(\text{bpy})_3]^{2+}$ on the electrode surface and therefore a higher concentration of $[\text{Ru}(\text{bpy})_3]^{2+}$ molecules to interact with methamphetamine. So, the system modified with nafion showed a linear working range of lower concentration of methamphetamine compared with that of the unmodified electrode system. LoD, LoQ and linear working range for MA are studied currently. Most ECL sensors have LoD of 10^{-9} to 10^{-8} (see Chapter3). the ECL intensity was found to be concentrations over the range of 5×10^{-8} to 2.5×10^{-4} mol/L. LoD for MA is 1.94×10^{-10} mol/L. The regression coefficient is 0.9931 for the experiment. Our system has very low detection limit and easy fabrication process. Our approach was applied in different medium such as saliva and human serum to detect MA. This technique is simple, rapid, selective and sensitive, and shows potential for the high- throughput quantitation of MA. The results show that the present electrochemical approach seems to provide a sensitive detection of MA in forensic applications. As future works, different films can be worked, and analytical parameters can be compared with our results. In this way, lower concentrations can be detected. Also, screen printed electrodes can be

used to have more portable device and analytical parameters can be compared with this study. More portable electrode system makes this study more suitable for commercial usage.

The biological fluid analysis for human serum and saliva and urine were carried out under the same condition. Due to some amino acids and proteins in serum, high background signal was observed. A dilution step was used to find optimum dilution rate. The serum and saliva were diluted with 0.1 M PBS. After the dilution process, three different concentration of MA were examined (100, 150 and 200 μM). % recovery of saliva and human serum were higher than %95 (see Tablo 3.1). Our study has very easy process and easy application and is time saving. There is no need for extraction and pre-treatment of sample. we used a basic dilution just to decrease the background signal. Average of % recovery of our study in serum as well in saliva was very similar to that of PBS study. % Recovery studies show that our study is suitable for real samples when compared with the results in the literature.

As a next step, we studied the ability of a simple nafion- $[\text{Ru}(\text{bpy})_3]^{2+}$ film modified electrode to detect MA alongside two of its metabolites (AMP and pOH-MA) within a variety of biological matrices. In this context, impact of pH on ECL analysis was examined and an optimum pH for analysis was observed as pH 9.0. There is a need for dilution of the body fluids because saliva is very viscose. Diffusion of the analytes is problem. A dilution phase was applied in this step to get rid of viscosity problem (see Chapter 4.4). Biological matrixes were diluted with 0.1 M PBS and it can be said that LoDs for MA and its metabolites are very sensitive, compared with the literature (see Tablo 4.1). At pH 9 LoDs for MA, AMP and pOH-MA in 0.1

M PBS are 74.6 nM, 1 μ M and 82.8 nM, respectively. When our analytical results with the literature, our modified ECL system can be used for real samples. Also, % recovery of MA, AMP and pOH-MA in body fluids are so high. Throughout the study, artificial body samples were used, and real street samples can be collected, and the samples are applied for the current modified electrode system and the results from artificial and real street samples can be compared.

To extend the working range of the current electrode system, different body samples can be examined such as hair, sweat. For hair samples, there must be a extraction phase to take liquid sample. That may a difference between both detection process. However, the results from both can be compared and usage of the current electrode system can be discussed.

Moreover, some interferences were worked and cocaine, diamorphine (blue) showed very high ECL intensity and those ECL signals are very similar to that of MA. nicotine (green) and paracetamol showed so low ECL intensity and that means that our system can detect MA in a street sample in which MA mixed with nicotine. That is important because most of street samples (MA) are generally diluted with nicotine. This interferences section can be extended and different components that are generally in real street samples can be worked, and selectivity of our modified system can be seen more obviously.

Unfortunately, the current approach (simple nafion-[Ru(bpy)₃]²⁺ film modified electrode) does not provide the ability to discriminate between the different ATS present, although it is possible to confidently determine that

at least one of these ATS is present. Determining the presence of an ATS is still extremely useful for screening or triage purposes for portable detection. This approach highlights the ability of this ECL based sensor to be applied to non-ideal biological matrices with little to no sample preparation and no extraction required, thereby being ideally suited for implementation within portable and on-site screening. This showcases the potential of ECL sensors for portable detection.

Next step was discrimination of structurally similar complexes. The aim of the work was to detect and distinguish MA, AMP, and p-OH-MA by using different Ir (III) complexes and osmium complexes on the modified electrode system. A mixed metal system such as ruthenium, iridium and osmium complex can be created and the individual luminophores work for the individual ATS for multi-analyte sensing. That modified electrode system can be used for body fluids and the concentration of MA, AMP and p-OH MA can be calculated separately by using only one electrode system. Iridium and osmium complexes were used to distinguish MA and its metabolites in samples. However, the modified electrode systems do not show ECL signal against the analytes. Different pH levels were worked to check impact of pH on ECL intensity. Obviously, It can be said that these metal complexes can not be utilised for distinguishing MA and its main metabolites within this current system but the metal complexes can be applied for detection of the ATS in different conditions or electrode systems in the future. Also, different novel luminophores can be synthesised and those metal complexes can be examined for distinguishing MA and its main metabolites.

Overall, the system modified with $[\text{Ru}(\text{bpy})_3]^{2+}$ is working for MA, AMP, and pOH-MA in aquatic medium and can detect those ATSs in body fluids with very low LoDs. The current system have high potential to be used for real samples and for commercial purpose. Iridium and osmium complexes used in the study do not show any ECL effect in our system but this section can be extended and more metal complex can be studied to distinguish the ATS.

7. References

1. Abbruscato, T. J., & Trippier, P. C. (2018). DARK Classics in Chemical Neuroscience: Methamphetamine. *ACS chemical neuroscience*.
2. Alsenedi, K. A., & Morrison, C. (2018). Determination of amphetamine-type stimulants (ATSS) and synthetic cathinones in urine using solid phase micro-extraction fibre tips and gas chromatography-mass spectrometry. *Analytical Methods*, 10(12), 1431-1440.
3. Aps, J. K., & Martens, L. C. (2005). The physiology of saliva and transfer of drugs into saliva. *Forensic science international*, 150(2-3), 119-131.
4. Arduini, F., Amine, A., Moscone, D., Ricci, F., & Palleschi, G. (2007). Fast, sensitive and cost-effective detection of nerve agents in the gas phase using a portable instrument and an electrochemical biosensor. *Analytical and bioanalytical chemistry*, 388(5-6), 1049-1057.
5. Asturias-Arribas, L., Alonso-Lomillo, M. A., Domínguez-Renedo, O., & Arcos-Martínez, M. J. (2013). Electrochemical determination of cocaine using screen-printed cytochrome P450 2B4 based biosensors. *Talanta*, 105, 131-134.
6. Richter, M. M. (2004). Electrochemiluminescence (ecl). *Chemical Reviews*, 104(6), 3003-3036.
7. Bakker, E.; Pretsch, E. (2005). Potentiometric sensors for trace-level analysis. *Trac-Trends in Analytical Chemistry*, 24(3), 199–207.

8. Balamurugan, S., Obubuafo, A., Soper, S. A., & Spivak, D. A. (2008). Surface immobilization methods for aptamer diagnostic applications. *Analytical and bioanalytical chemistry*, 390(4), 1009-1021.
9. Balbino, M. A., de Menezes, M. M. T., Eleoterio, I. C., Saczk, A. A., Okumura, L. L., Tristao, H. M., & de Oliveira, M. F. (2012). Voltammetric determination of Δ^9 -THC in glassy carbon electrode: An important contribution to forensic electroanalysis. *Forensic science international*, 221(1-3), 29-32.
10. Bard, A. J., & Faulkner, L. R. (2001). Fundamentals and applications. *Electrochemical Methods*, 2, 482.
11. Beckett, A. H., Boyes, R. N., & Tucker, G. T. (1968). Use of the analogue computer to examine the quantitative relation between urinary pH and kidney reabsorption of drugs partially ionized at physiological pH. *Journal of Pharmacy and Pharmacology*, 20(4), 269-276.
12. Berman, S., O'Neill, J., Fears, S., Bartzokis, G., & London, E. D. (2008). Abuse of amphetamines and structural abnormalities in the brain. *Annals of the New York Academy of Sciences*, 1141(1), 195-220.
13. Bramness, J. G., Reid, M. J., Solvik, K. F., & Vindenes, V. (2015). Recent trends in the availability and use of amphetamine and methamphetamine in Norway. *Forensic science international*, 246, 92-97.
14. Brown, S. D., & Melton, T. C. (2011). Trends in bioanalytical methods for the determination and quantification of club drugs: 2000–2010. *Biomedical chromatography*, 25(1-2), 300-321.

15. Buerk, D. (1993). *Biosensors. Theory and Applications*. Technomic Publishing, Lancaster.
16. Chaubey, A., & Malhotra, B. (2002). Mediated biosensors. *Biosensors and bioelectronics*, 17(6-7), 441-456.
17. Christian, G.D., (2004). *Analytical Chemistry*, John Wiley & Sons, USA.
18. Sloop, G., Hall, M., Simmons, G. T., & Robinson, C. A. (1995). False-positive postmortem EMIT drugs-of-abuse assay due to lactate dehydrogenase and lactate in urine. *Journal of analytical toxicology*, 19(7), 554-556.
19. Cole, M. D. (2006). *The Analysis of Drugs of Abuse: An Instruction Manual: An Instruction Manual*. CRC Press.
20. Crouch, D. J. (2005). Oral fluid collection: the neglected variable in oral fluid testing. *Forensic science international*, 150(2-3), 165-173.
21. Cruickshank, C. C., & Dyer, K. R. (2009). A review of the clinical pharmacology of methamphetamine. *Addiction*, 104(7), 1085-1099.
22. de Cássia Mariotti, K., et al. (2014). Simultaneous determination of fenproporex, diethylpropione and methylphenidate in oral fluid by LC-MS/MS. *Chromatographia*, 77(1-2), 83-90.
23. Gao, W., Jeanneret, S., Yuan, D., Cherubini, T., Wang, L., Xie, X., & Bakker, E. (2019). Electrogenerated chemiluminescence for chronopotentiometric sensors. *Analytical chemistry*, 91(7), 4889-4895.

24. Deventer, K., Van Eenoo, P., & Delbeke, F. T. (2006). Screening for amphetamine and amphetamine-type drugs in doping analysis by liquid chromatography/mass spectrometry. *Rapid communications in mass spectrometry*, 20(5), 877-882.

25. Doeven, E. H., Barbante, G. J., Harsant, A. J., Donnelly, P. S., Connell, T. U., Hogan, C. F., & Francis, P. S. (2015). Mobile phone-based electrochemiluminescence sensing exploiting the 'USB On-The-Go' protocol. *Sensors and Actuators B: Chemical*, 216, 608-613.

26. Eggins, B. (2002). Chemical sensors and biosensors. Analytical Techniques in the Sciences. John Wiley & Sons, West Sussex.

27. Evans, D. H., O'Connell, K. M., Petersen, R. A., & Kelly, M. J. (1983). Cyclic voltammetry.

28. Factor, B., Muegge, B., Workman, S., Bolton, E., Bos, J., & Richter, M. M. (2001). Surfactant Chain Length Effects on the Light Emission of Tris (2, 2 '-bipyridyl) ruthenium (II)/Tripropylamine Electrogenerated Chemiluminescence. *Analytical chemistry*, 73(19), 4621-4624.

29. Fishbein, D. H., & Pease, S. E. (1996). *The dynamics of drug abuse*. Allyn & Bacon.

30. Foppe, K. S., Hammond-Weinberger, D. R., & Subedi, B. (2018). Estimation of the consumption of illicit drugs during special events in two communities in Western Kentucky, USA using sewage epidemiology. *Science of The Total Environment*, 633, 249-256.

- 31.** Forster, R. J., Bertoncello, P., & Keyes, T. E. (2009). Electrogenated chemiluminescence. *Annual Review of Analytical Chemistry*, 2, 359-385.
- 32.** Truong, C. K. P., Nguyen, T. D. D., & Shin, I. S. (2019). Electrochemiluminescent chemosensors for clinical applications: A review. *BioChip Journal*, 13(3), 203-216.
- 33.** Garrido, E. M. P. J., Garrido, J. M. P. J., Milhazes, N., Borges, F., & Oliveira-Brett, A. M. (2010). Electrochemical oxidation of amphetamine-like drugs and application to electroanalysis of ecstasy in human serum. *Bioelectrochemistry*, 79(1), 77-83.
- 34.** Grafinger, K. E., Liechti, M. E., & Liakoni, E. (2020). Clinical value of analytical testing in patients presenting with new psychoactive substances intoxication. *British journal of clinical pharmacology*, 86(3), 429-436.
- 35.** Gooch, J., Daniel, B., Parkin, M., & Frascione, N. (2017). Developing aptasensors for forensic analysis. *TrAC Trends in Analytical Chemistry*, 94, 150-160.
- 36.** Graziano, S., Anzillotti, L., Mannocchi, G., Pichini, S., & Busardò, F. P. (2019). Screening methods for rapid determination of new psychoactive substances (NPS) in conventional and non-conventional biological matrices. *Journal of pharmaceutical and biomedical analysis*, 163, 170-179.
- 37.** Gu, M. B. (2016). *Biosensors based on aptamers and enzymes*. H. S. Kim (Ed.). Springer.

38. Huang, R., Wu, M., Goldman, M. J., & Li, Z. (2015). Encapsulation of enzyme via one-step template-free formation of stable organic–inorganic capsules: A simple and efficient method for immobilizing enzyme with high activity and recyclability. *Biotechnology and bioengineering*, 112(6), 1092-1101.

39. Hulanicki, A., Glab, S., & Ingman, F. (1989). IUPAC discussion paper Commission V. 1.

40. Holze, R. (2009). RG Compton and CE Banks, Understanding cyclic voltammetry.

41. Home Office. Drug Abuse: Findings from the 2013/14 Crime Survey for England and Wales, [online], [cited 21st June 2018], Available from <https://www.gov.uk/government/publications/drug-misuse-findings-from-the-2013-to-2014-csew/drug-misuse-findings-from-the-201314-crime-survey-for-england-and-wales>

42. Home Office: Misuse of Drugs Regulations 2001. [online], [cited 21st June 2018], Available from http://www.legislation.gov.uk/ukxi/2001/3998/pdfs/ukxi_20013998_en.pdf

43. Home Office: Misuse of Drugs Act 1971, [online], [cited 21st June 2018], [last updated 2014], Available from <http://www.legislation.gov.uk/ukpga/1971/38/contents>.

44. Kankaanpää, A., Gunnar, T., Ariniemi, K., Lillsunde, P., Mykkänen, S., & Seppälä, T. (2004). Single-step procedure for gas chromatography–mass spectrometry screening and quantitative determination of amphetamine-type stimulants and related drugs in blood,

serum, oral fluid and urine samples. *Journal of Chromatography B*, 810(1), 57-68.

45. Karunakaran, C., Rajkumar, R., & Bhargava, K. (2015). Introduction to Biosensors. In *Biosensors and Bioelectronics* (pp. 1-68).

46. Katch, R. K., Smith, M. S., LaBotz, M., Belval, L. N., & Hosokawa, Y. (2018). Drugs and Supplements. In *Sport and Physical Activity in the Heat* (pp. 347-365). Springer, Cham.

47. Katz, E., & Halámek, J. (2016). Forensic Science—Chemistry, Physics, Biology, and Engineering—Introduction. *Forensic Science: A Multidisciplinary Approach*, 1.

48. Kim, J. Y., Cheong, J. C., Kim, M. K., Lee, J. I., & In, M. K. (2008). Simultaneous determination of amphetamine-type stimulants and cannabinoids in fingernails by gas chromatography-mass spectrometry. *Archives of pharmacal research*, 31(6), 805-813.

49. Kim, D., & Herr, A. E. (2013). Protein immobilization techniques for microfluidic assays. *Biomicrofluidics*, 7(4), 041501.

50. Kimmel, D. W., LeBlanc, G., Meschievitz, M. E., & Clifffel, D. E. (2011). Electrochemical sensors and biosensors. *Analytical chemistry*, 84(2), 685-707.

51. Kirsch J, Siltanen C, Zhou Q, Revzin A and Simonian A 2013 Biosensor technology: recent advances in threat agent detection and medicine *Chem. Soc. Rev.* 42 8733–68

- 52.** Liu, D. M., Chen, J., & Shi, Y. P. (2018). Advances on methods and easy separated support materials for enzymes immobilization. *TrAC Trends in Analytical Chemistry*.
- 53.** Lu, M. C., & Whang, C. W. (2004). The role of direct oxalate oxidation in electrogenerated chemiluminescence of poly (4-vinylpyridine)-bound Ru (bpy) $2\text{Cl}^+/\text{oxalate}$ system on indium tin oxide electrodes. *Analytica chimica acta*, 522(1), 25-33.
- 54.** McGeehan, J., & Dennany, L. (2016). Electrochemiluminescent detection of methamphetamine and amphetamine. *Forensic science international*, 264, 1-6.
- 55.** Maurer, M., Pronk, W., & Larsen, T. A. (2006). Treatment processes for source-separated urine. *Water research*, 40(17), 3151-3166.
- 56.** Merz, F. (2017). United Nations Office on Drugs and Crime: World Drug Report 2017. *SIRIUS-Zeitschrift für Strategische Analysen*, 2(1), 85-86.
- 57.** Miao, W.J., (2008). *Electrogenerated chemiluminescence and its biorelated applications*. Chemical Reviews, **108**(7): p. 2506-2553.
- 58.** Mirsky, V. M., Riepl, M., & Wolfbeis, O. S. (1997). Capacitive monitoring of protein immobilization and antigen-antibody reactions on monomolecular alkylthiol films on gold electrodes. *Biosensors & Bioelectronics*, 12(9-10), 977–989.
- 59.** Miyaguchi, H., & Inoue, H. (2011). Determination of amphetamine-type stimulants, cocaine and ketamine in human hair by

liquid chromatography/linear ion trap–Orbitrap hybrid mass spectrometry. *Analyst*, 136(17), 3503-3511.

60. Moffat, A. C. (2000). Drugs of abuse. *Science & Justice*, 40(2), 89-92.

61. Moeller, K. E., Lee, K. C., & Kissack, J. C. (2008, January). Urine drug screening: practical guide for clinicians. In *Mayo Clinic Proceedings* (Vol. 83, No. 1, pp. 66-76). Elsevier.

62. White, R. M., & Moore, C. M. (2018). *Detection of Drugs and Their Metabolites in Oral Fluid*. Elsevier.

63. Dennany, L., O'Reilly, E. J., Innis, P. C., Wallace, G. G., & Forster, R. J. (2008). The influence of poly (2-methoxyaniline-5-sulfonic acid) on the electrochemical and photochemical properties of a highly luminescent ruthenium complex. *Electrochimica acta*, 53(13), 4599-4605.

64. Rusmini, F., Zhong, Z., & Feijen, J. (2007). Protein immobilization strategies for protein biochips. *Biomacromolecules*, 8(6), 1775-1789.

65. Shaw, L., & Dennany, L. (2017). Applications of electrochemical sensors: Forensic drug analysis. *Current Opinion in Electrochemistry*, 3(1), 23-28.

66. Schaefer, N., Peters, B., Schmidt, P., & Ewald, A. H. (2013). Development and validation of two LC-MS/MS methods for the detection and quantification of amphetamines, designer amphetamines, benzoylecgonine, benzodiazepines, opiates, and opioids in urine using

turbulent flow chromatography. *Analytical and bioanalytical chemistry*, 405(1), 247-258.

67. Schaudies, R. P. (2014). *Biological Identification: DNA Amplification and Sequencing, Optical Sensing, Lab-on-chip and Portable Systems*. Elsevier.

68. Scholz, F. (2010). *Electroanalytical methods* (Vol. 1). Berlin-Heidelberg: Springer.

69. Shan, Y., J.J. Xu, and H.Y. Chen. (2011). *Enhanced electrochemiluminescence quenching of CdS:Mn nanocrystals by CdTe QDs-doped silica nanoparticles for ultrasensitive detection of thrombin*. *Nanoscale*, 3(7): p. 2916-2923.

70. Sharma, A., & Rogers, K. R. (1994). Biosensors. *Measurement Science and Technology*, 5(5), 461.

71. Shrivastava, A., & Gupta, V. (2011). Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of young scientists*, 2(1), 21-21.

72. Slepchenko, G. B., Gindullina, T. M., & Nekhoroshev, S. V. (2017). Capabilities of the electrochemical methods in the determination of narcotic and psychotropic drugs in forensic chemistry materials. *Journal of Analytical Chemistry*, 72(7), 703-709.

73. Sparkman, O. D., Penton, Z., & Kitson, F. G. (2011). *Gas chromatography and mass spectrometry: a practical guide*. Academic Press.

74. Stout, P. R. (2009). Forensic Toxicology. In *Information Resources in Toxicology (Fourth Edition)* (pp. 293-296).
75. Tao, Y. U. A. N. (2011). 2, LIU Zhong-Yuan¹, HU Lian-Zhe^{1, 2}, XU Guo-Bao¹ ¹ (State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022) ² (Graduate University of Chinese Academy of Sciences, Beijing 100039); Electrochemical and Electrochemiluminescent Aptasensors [J]. *Chinese Journal of Analytical Chemistry*, 7.
76. Thevenot, D. R., Toth, K., Durst, R. A., & Wilson, G. S. (1999). Electrochemical biosensors: recommended definitions and classification. *Pure and applied chemistry*, 71(12), 2333-2348.
77. Thompson, L. K., & Cone, E. J. (1987). Determination of δ 9-tetrahydrocannabinol in human blood and saliva by high-performance liquid chromatography with amperometric detection. *Journal of Chromatography B: Biomedical Sciences and Applications*, 421, 91-97.
78. Thompson, R. B., Gryczynski, I., & Malicka, J. (2002). Fluorescence polarization standards for high-throughput screening and imaging. *BioTechniques*, 32(1), 34-42.
79. Tombelli, S., Minunni, M., & Mascini, M. (2007). Aptamers-based assays for diagnostics, environmental and food analysis. *Biomolecular engineering*, 24(2), 191-200.
80. Tunstall, B. J., Ho, C. P., Cao, J., Vendruscolo, J. C., Schmeichel, B. E., Slack, R. D. and Koob, G. F. (2018). Atypical dopamine

transporter inhibitors attenuate compulsive-like methamphetamine self-administration in rats. *Neuropharmacology*, 131, 96-103.

81. Yao, Y., Li, H., Wang, D., Liu, C., & Zhang, C. (2017). An electrochemiluminescence cloth-based biosensor with smartphone-based imaging for detection of lactate in saliva. *Analyst*, 142(19), 3715-3724.

82. Vearrier, D., Greenberg, M. I., Miller, S. N., Okaneku, J. T., & Haggerty, D. A. (2012). Methamphetamine: history, pathophysiology, adverse health effects, current trends, and hazards associated with the clandestine manufacture of methamphetamine. *Disease-a-Month*, 58(2), 38-89. H.H. Maurer, Hyphenated mass spectrometric techniques – indispensable tools in clinical and forensic toxicology and in doping control, *J. Mass. Spectrom*, 2006, **41**, 1399-1413.

83. Wang, J. (2006). *Analytical electrochemistry*. John Wiley & Sons.

84. Xu, Z., Yu, J., & Liu, G. (2013). Fabrication of carbon quantum dots and their application for efficient detecting Ru (bpy)₃²⁺ in the solution. *Sensors and Actuators B: Chemical*, 181, 209-214.

85. Yang, H., Li, X., Batchelor-McAuley, C., Sokolov, S. V., Kätelhön, E., & Compton, R. G. (2017). Immobilised Electrocatalysts: Nafion Particles Doped with Ruthenium (II) Tris (2, 2'-bipyridyl). *Chemistry–A European Journal*, 23(69), 17605-17611.

86. Yang, Y. N., Lin, H. I., Wang, J. H., Shiesh, S. C., & Lee, G. B. (2009). An integrated microfluidic system for C-reactive protein measurement. *Biosensors and Bioelectronics*, 24(10), 3091-3096.

87. Yarbakht, M., & Nikkhah, M. (2016). Unmodified gold nanoparticles as a colorimetric probe for visual methamphetamine detection. *Journal of Experimental Nanoscience*, 11(7), 593-601.
88. Yao, W., Wang, L., Wang, H., Zhang, X., & Li, L. (2009). An aptamer-based electrochemiluminescent biosensor for ATP detection. *Biosensors and Bioelectronics*, 24(11), 3269-3274.
89. Yin, X. B., Dong, S., & Wang, E. (2004). Analytical applications of the electrochemiluminescence of tris (2, 2'-bipyridyl) ruthenium and its derivatives. *TrAC Trends in Analytical Chemistry*, 23(6), 432-441.
90. B., Dong, S., & Wang, E. (2004). Analytical applications of the electrochemiluminescence of tris (2, 2'-bipyridyl) ruthenium and its derivatives. *TrAC Trends in Analytical Chemistry*, 23(6), 432-441.
91. Zheng, H. and Y. Zu, *Emission of Tris(2,2'-bipyridine)ruthenium(II) by Coreactant Electrogenerated Chemiluminescence: From O₂-Insensitive to Highly O₂-Sensitive*. The Journal of Physical Chemistry B, 2005. **109**(24): p. 12049-12053.
92. World Drug Report 2020 (United Nations publication, Sales No. E.20.XI.6)
93. Participation, E. (1979, January 31). *Misuse of Drugs Act 1971*. Legislation.gov.uk.
<https://www.legislation.gov.uk/ukpga/1971/38/contents>.
94. Woźniak, M. K., Wiergowski, M., Aszyk, J., Kubica, P., Namieśnik, J., & Biziuk, M. (2018). Application of gas chromatography–tandem mass spectrometry for the determination of amphetamine-type

stimulants in blood and urine. *Journal of pharmaceutical and biomedical analysis*, 148, 58-64.

95. Janata, J. (2008). Introduction: Modern topics in chemical sensing. *Chemical reviews*, 108(2), 327-328.

96. Mokhtarzadeh, A., Tabar zad, M., Ranjbari, J., de la Guardia, M., Hejazi, M., & Ramezani, M. (2016). Aptamers as smart ligands for nano-carriers targeting. *TrAC Trends in Analytical Chemistry*, 82, 316-327.

97. Mauritz, K. A., & Moore, R. B. (2004). State of understanding of Nafion. *Chemical reviews*, 104(10), 4535-4586.

98. Kanoufi, F., & Bard, A. J. (1999). Electrogenerated chemiluminescence. 65. An investigation of the oxidation of oxalate by tris (polypyridine) ruthenium complexes and the effect of the electrochemical steps on the emission intensity. *The Journal of Physical Chemistry B*, 103(47), 10469-10480.

99. Brooks, S. C., Vinyard, D. J., & Richter, M. M. (2006). Electrogenerated chemiluminescence of (bis-bipyridyl) ruthenium (II) acetylacetonate complexes. *Inorganica chimica acta*, 359(14), 4635-4638.

100. Zu, Y., & Bard, A. J. (2001). Electrogenerated chemiluminescence. 67. Dependence of light emission of the tris (2, 2 ') bipyridylruthenium (II)/tripropylamine system on electrode surface hydrophobicity. *Analytical chemistry*, 73(16), 3960-3964.

101. Li, F., & Zu, Y. (2004). Effect of nonionic fluorosurfactant on the electrogenerated chemiluminescence of the tris (2, 2 ' -bipyridine)

ruthenium (II)/Tri-n-propylamine system: lower oxidation potential and higher emission intensity. *Analytical chemistry*, 76(6), 1768-1772.

102. Dennany, L., O'Reilly, E. J., Keyes, T. E., & Forster, R. J. (2006). Electrochemiluminescent monolayers on metal oxide electrodes: detection of amino acids. *Electrochemistry communications*, 8(10), 1588-1594.

103. Dennany, L., Hogan, C. F., Keyes, T. E., & Forster, R. J. (2006). Effect of surface immobilization on the electrochemiluminescence of ruthenium-containing metallopolymer. *Analytical chemistry*, 78(5), 1412-1417.

104. Zhang, L., Guo, Z., Xu, Z., & Dong, S. (2006). Highly sensitive electrogenerated chemiluminescence produced at Ru (bpy) 32^+ in Eastman-AQ55D-carbon nanotube composite film electrode. *Journal of Electroanalytical Chemistry*, 592(1), 63-67.

105. Muegge, B. D., & Richter, M. M. (2005). Electrogenerated chemiluminescence from polymer-bound ortho-metallated iridium (III) systems. *Luminescence: the journal of biological and chemical luminescence*, 20(2), 76-80.

106. Kim, J. I., Shin, I. S., Kim, H., & Lee, J. K. (2005). Efficient electrogenerated chemiluminescence from cyclometalated iridium (III) complexes. *Journal of the American Chemical Society*, 127(6), 1614-1615.

107. Creutz, C., Chou, M., Netzel, T. L., Okumura, M., & Sutin, N. (1980). Lifetimes, spectra, and quenching of the excited states of

polypyridine complexes of iron (II), ruthenium (II), and osmium (II). *Journal of the American Chemical Society*, 102(4), 1309-1319.

108. Bruce, D., Richter, M. M., & Brewer, K. J. (2002). Electrochemiluminescence from Os (phen) ₂ (dppene) ²⁺(phen= 1, 10-phenanthroline and dppene= bis (diphenylphosphino) ethene). *Analytical chemistry*, 74(13), 3157-3159.

109. Gorman, B. A., Francis, P. S., & Barnett, N. W. (2006). Tris (2, 2'-bipyridyl) ruthenium (II) chemiluminescence. *Analyst*, 131(5), 616-639.

110. Aboul-Enein, H. Y., Stefan, R. I., van Staden, J. F., Zhang, X. R., Garcia-Campana, A. M., & Baeyens, W. R. (2000). Recent developments and applications of chemiluminescence sensors. *Critical Reviews in Analytical Chemistry*, 30(4), 271-289.

111. Ligler, F. S., & Taitt, C. R. (Eds.). (2002). *Optical biosensors: present & future*. Gulf Professional Publishing.

112. Barbante, G. J., Hogan, C. F., Wilson, D. J., Lewcenko, N. A., Pfeffer, F. M., Barnett, N. W., & Francis, P. S. (2011). Simultaneous control of spectroscopic and electrochemical properties in functionalised electrochemiluminescent tris (2, 2'-bipyridine) ruthenium (ii) complexes. *Analyst*, 136(7), 1329-1338.

113. Liu, Z., Qi, W., & Xu, G. (2015). Recent advances in electrochemiluminescence. *Chemical Society Reviews*, 44(10), 3117-3142.

- 114.** Kapturkiewicz, A. (2016). Cyclometalated iridium (III) chelates—a new exceptional class of the electrochemiluminescent luminophores. *Analytical and bioanalytical chemistry*, 408(25), 7013-7033.
- 115.** Zysman-Colman, E. (Ed.). (2017). *Iridium (III) in optoelectronic and photonics applications*. John Wiley & Sons.
- 116.** Chen, L., Hayne, D. J., Doeven, E. H., Agugiaro, J., Wilson, D. J., Henderson, L. C., ... & Francis, P. S. (2019). A conceptual framework for the development of iridium (III) complex-based electrogenerated chemiluminescence labels. *Chemical science*, 10(37), 8654-8667.
- 117.** Zhou, Y., Li, W., Yu, L., Liu, Y., Wang, X., & Zhou, M. (2015). Highly efficient electrochemiluminescence from iridium (III) complexes with 2-phenylquinoline ligand. *Dalton Transactions*, 44(4), 1858-1865.
- 118.** Zhou, Y., Gao, H., Wang, X., & Qi, H. (2015). Electrogenerated chemiluminescence from heteroleptic iridium (III) complexes with multicolor emission. *Inorganic chemistry*, 54(4), 1446-1453.
- 119.** Hindson, C. M., Hanson, G. R., Francis, P. S., Adcock, J. L., & Barnett, N. W. (2011). Any old radical won't do: an EPR study of the selective excitation and quenching mechanisms of [Ru (bipy) ₃]²⁺ chemiluminescence and electrochemiluminescence. *Chemistry—A European Journal*, 17(29), 8018-8022.
- 120.** Takahashi, F., Nitta, S., Shimizu, R., & Jin, J. (2018). Electrochemiluminescence and voltammetry of tris (2, 2'-bipyridine) ruthenium (II) with amphetamine-type stimulants as coreactants: An

application to the discrimination of methamphetamine. *Forensic Toxicology*, 36(1), 185-191.

121. Shin, I. S., Kang, Y. T., Lee, J. K., Kim, H., Kim, T. H., & Kim, J. S. (2011). Evaluation of electrogenerated chemiluminescence from a neutral Ir (III) complex for quantitative analysis in flowing streams. *Analyst*, 136(10), 2151-2155.