ELECTROCHEMICAL DETECTION OF COCAINE ON SCREEN PRINTED ELECTRODES

TARGET ANALYTE-ASSISTED SENSITIVE ELECTROCHEMICAL DETECTION OF COCAINE USING SCREEN PRINTED ELECTRODES

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Lay Abstract

Cocaine is one of the most abused drugs worldwide causing various health complications and death due to overconsumption. The detection of cocaine in biological fluid or hair is often a complicated and expensive procedure that is time-consuming. The use of electrochemical techniques allows for the rapid detection of cocaine. By focusing on using saliva, the process is facilitated given that it is the easiest fluid to collect from individuals. In this thesis, a new method of cocaine detection in saliva is presented using electrochemistry and modification of the device to detect very low concentrations of the illicit substance. The combined use of electrochemical techniques and machine learning resulted in the successful development of a method to detect very low concentrations of cocaine in saliva.

Abstract

Cocaine is one of the most abused drugs worldwide, with over 20 million users in 2019. The use of cocaine leads to various health conditions and death due to overconsumption. Existing detection technologies fall short of meeting the guidelines set by organizations regarding a practical roadside cocaine detection device. Herein, a novel electrochemical cocaine detection method is presented using the electroactive characteristics of the cocaine molecule that does not require any biomolecules or chemicals for detection. This research implements the cocaine-modified carbon working electrodes to detect cocaine using Cyclic Voltammetry in phosphate buffer solution and human saliva. The affinity of the cocaine samples to the cocaine-modified screen printed electrodes is demonstrated with the support and implementation of machine learning. The proposed cocaine detection was used to detect concentrations from 10 to 1000 ng/mL. Furthermore, the successful detection of cocaine in the presence of various interfering substances revealed that the present study is highly specific and promising for developing a roadside oral fluid cocaine detection kit.

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List of Abbreviations

Cocaine	COC
Limit of Detection	LoD
Square Wave Voltammetry	SWV
Cyclic Voltammetry	CV
Random Forest	RF
Decision Tree	DT
Support Vector Machine	SVM
Pristine/bare/unmodified electrode	Z-P
X-ray photoelectron spectroscopy	XPS
Modified electrode	m-Z-COC
Electronic tongue	ET
Machine Learning	ML
Gold nanoparticles	AuNPs
(−)-trans-Δ^9-tetrahydrocannabinol	THC
Roadside Testing Assessment	ROSITA
3,3’,5,5'-Tetramethylbenzidine	TMB
Methanol	MetOH
Gaussian Radial Basis Function	RBF
Oral fluid	OF
Phosphate Buffer Solution	PBS
Ecgonine Methyl Ester	EME
Screen printed electrodes	SPE
Driving Under the Influence of Drugs, Alcohol and Medicines	DRUID
The International Union of Pure and Applied Chemistry	IUPAC

Machine Learning	ML
Counter Electrode	CE
Binding Energy	BE
Transmission Electron Microscopy	TEM
Electronic Tongue	ET
Reference Electrode	RE
Working Electrode	WE
Benzoylecgonine	BZE
Norcocaine	NC

Chapter 1: Introduction

1.1 Motivation

The use and traffic of illicit substances is a worldwide issue with tremendous consequences on the environment, criminal activity, and consumers' health. Recently, the use of saliva to detect illicit substances has been explored since it is a much easier medium to collect and undoubtedly less invasive than blood or urine. Specifically, the electrochemical detection of illicit substances in oral fluid has proven to be highly accurate and takes under a minute to analyze. The current protocol for detecting illicit drugs in blood or urine involves the use of methods such as mass spectrometry and gas chromatography due to their reliability. However, these methods are costly and not practical for roadside use as they are enormous and require trained personnel to be operated. There is a high demand for a roadside device capable of detecting illicit substances. Much like breathalyzers, this device should be minimally invasive and produce results within minutes. Using electrochemistry makes this application possible.

1.2 Objectives

The main objective of this thesis is to develop an electrochemical sensor that can detect various concentrations of cocaine in PBS and human saliva. This sensor will detect cocaine accurately and rapidly without the need for highly trained personnel and expensive mechanisms. This objective has been pursued using the electroactive nature of the cocaine

molecule and various electrochemical techniques that complement the detection process by developing a signal from which the correlation between its intensity and the concentrations being analyzed can be obtained. To achieve this, several components were explored including the challenges faced when sensing in oral fluid as well as the different parameters to be optimized when creating a sensor that has high affinity towards the target molecule.

1.3 Thesis Outline

The remainder of this thesis is arranged as follows:

Chapter 2 includes literature review of the metabolism of cocaine, the detection of cocaine, and a general introduction to electrochemical sensors.

Chapter 3 focuses on the use of saliva as the biological testing matrix. Collecting devices and methods are discussed. Obstacles that are encountered when using saliva in electrochemical sensing are also presented.

Chapter 4 presents the electrochemical detection of cocaine in PBS. The electrode optimization steps are outlined in detail and the steps taken to achieve a low detection limit using a biomolecule free approach is presented.

Chapter 5 focuses on the results from the investigation approach presented in chapter four but using real human saliva instead of PBS. This chapter focuses on the electrochemical detection of cocaine in saliva as well as the challenges

overcome using machine learning.

Chapter 6 summarizes the conclusions of this work by providing a summary of the work, contributions to the field, and potential future work.

Chapter 2: Literature Review: Cocaine and the Electrochemical Detection of the Drug

2.1 Preface

The literature review is intended to explain the effects of cocaine on the human body. The pharmacokinetics and metabolism of cocaine in the human body are discussed. The elimination of cocaine in the body, as well as the correlation between different biological fluids is discussed.

Ana Gomez Cardoso is the sole author of this section.

2.2 Introduction to Cocaine Pharmacokinetics and Metabolism

Discussions on cocaine's pharmacokinetics in saliva is a well-researched area with several different opinions and outcomes. Pharmacokinetic studies of cocaine (COC) are used to determine the concentration of COC following the administration of known doses to assess the required sensitivity of oral fluid (OF) detecting devices. Three pathways metabolize COC in humans:

- Hydrolysis into ecgonine methyl ester (EME) by butyrylcholinesterase
- Hydrolysis into benzoylecgonine (BZE) by carboxylesterase or spontaneously
- De-methylation by liver microsomal cytochrome generating norcocaine (NC) [1].

Though only three were mentioned, several other smaller metabolites are involved in the process, and all are inactive in the human body [1]. Less than 5% of the administered dose of COC is excreted unmetabolized in urine [2]. Although COC is eliminated rapidly, it has

been suggested by several researchers that the chronic use of the drug can result in accumulation and prolonged eliminations even while abstaining from use [3]. Coe et al. [3] studied the elimination of COC and its metabolites in urine, plasma, and OF in individuals who reported a history of COC abuse. Volunteers in the study were housed in a clinical ward and orally administered up to 16 doses of COC daily. Carryover of COC was evident in some subjects, and the authors found consistently higher levels of the drug in the brain and body fat in those individuals who were a part of the chronically treated group.

Schepers et al. [4] performed a similar experiment, and both groups concluded that the regular use of COC alters the elimination of the drug from the human body. While COC can be detected in OF within minutes of administration, it is also rapidly eliminated with a half-life ranging from 1.1 – 3.8 hours [5]. However, it was demonstrated by Schepers et al. [4] that individuals who frequently abused the drug had unmetabolized COC in OF through the first 24 h and in urine for up to 5 days following administration. A more in-depth study of the metabolites further explains the drug's pharmacokinetics. The passage of compounds from plasma to OF occurs through passive diffusion. This action depends on the drug pKa, weight, structure, salivary and plasma pH, and flow rate [6]. Though some studies have found significant correlations between the concentration of COC in plasma, urine, and OF (P/U/OF) [6], [7] contradicting reports have been published where authors comment on the low correlation between P/U/OF due to the different modes of drug administration. Coe et al. [3] reported that intravenous COC administration resulted in a COC plasma elimination half-life from 0.26-3.44 h and 0.55-4.79 after intranasal administration. However, the ratio between plasma and OF ranged from 0.36-9.74 among individuals. Although Coe et al. [3]

do not attribute the differing ratios to the mode of administration, other researchers such as Schepers et al. [4] have reported the possibility of this being a significant factor.

2.3 Direct and Disposable Electrochemical Sensing of Analytes

The idea of screen-printing came from the microelectronics industry, which offers large-scale production of inexpensive and reliable electrodes designed for onsite monitoring [8]. In application, electrodes are purchased in high volume by research groups or made in-house by research teams. Commonly used substrates include glass, ceramic, or plastic because they are insulating materials [9]. SPE is composed of three electrodes: the working electrode (WE), the reference electrode (RE) and the counter electrode (CE) [10]. The WE and CE are mainly composed of inexpensive materials such as carbon or carbon modifications [11]. The RE is mainly composed of silver/silver chloride [9].

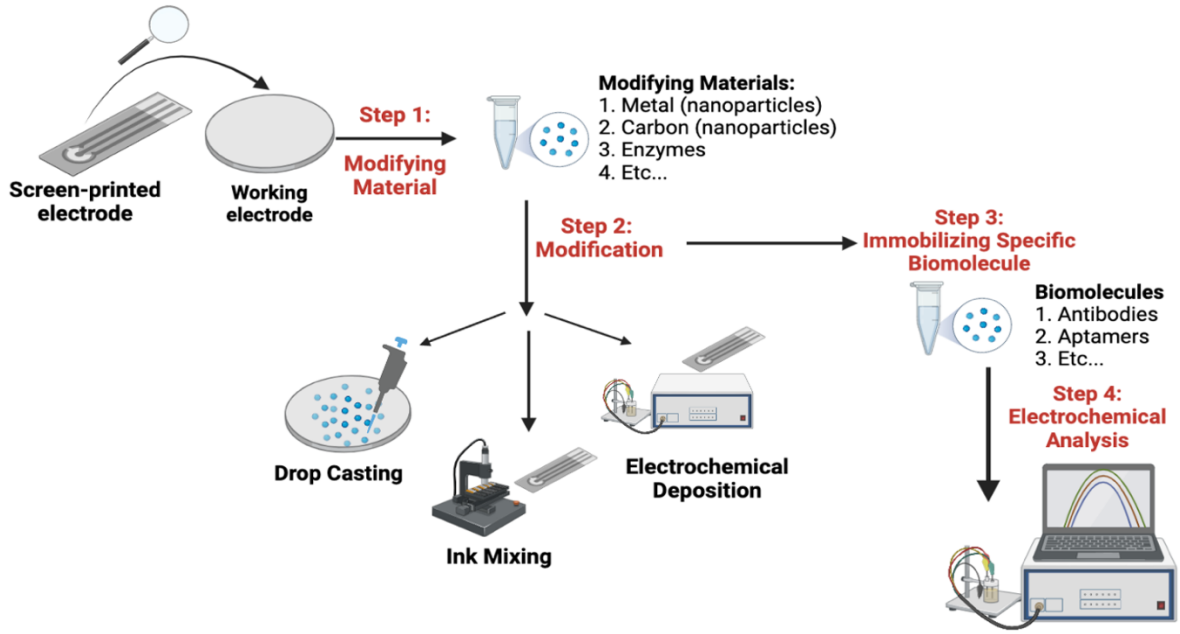

Figure 2-1: Process of modifying SPE using Step 1: several different materials affect the surface. Step 2: Modification can be done in several ways, including electrodeposition,

ink mixing, and drop-casting. Step 3: Immobilizing the scavenging agents depending on the biomarker of choice. Step 4: Signal analysis.

Although it is possible, few reports of SPE being used without any modification [12], [13]. Usually, the analytes of choice can only be detected at high concentrations, and on direct SPE, the limits of detection (LoD) are not desirable for real-life applications. Most commonly, SPE are modified with an array of substances and materials, including enzymes, polymers, antibodies, and nucleic acids [10].

2.3.1 Recent Development of Biosensing and Analytical Cocaine Determination

The biomolecule-mediated detection of COC involves using antibodies, aptamers, or enzymes [14], [15]. These methods are often paired with the help of nanomaterials to enhance the mechanism's colorimetric or electrochemical abilities [16], [17]. Colorimetry has been explored as a mechanism used to detect COC. Sanli et al. [18] developed a colorimetric aptasensor functionalized with gold nanoparticles (AuNPs) (Figure 2-2 A). The sensor has a low LOD of 0.2 nM (67.96 ng/mL) and demonstrated excellent results using OF as the testing matrix. Although the biosensor was highly specific and selective, the total testing time was approximately 90 minutes which is not practical for roadside applications. Ghorbanizamani et al. [19] also employed the use of colorimetry for the detection of COC in OF. The team created a colorimetric lateral flow immunoassay with a LOD of 0.49 ng/mL and 97% accuracy. As seen in Sanli et al. [18], these biosensors require incubation times. In the case of Ghorbanizamani et al. [19], the results are qualitative, which provides no information on the amount consumed by the individual (Figure 2-2 B).

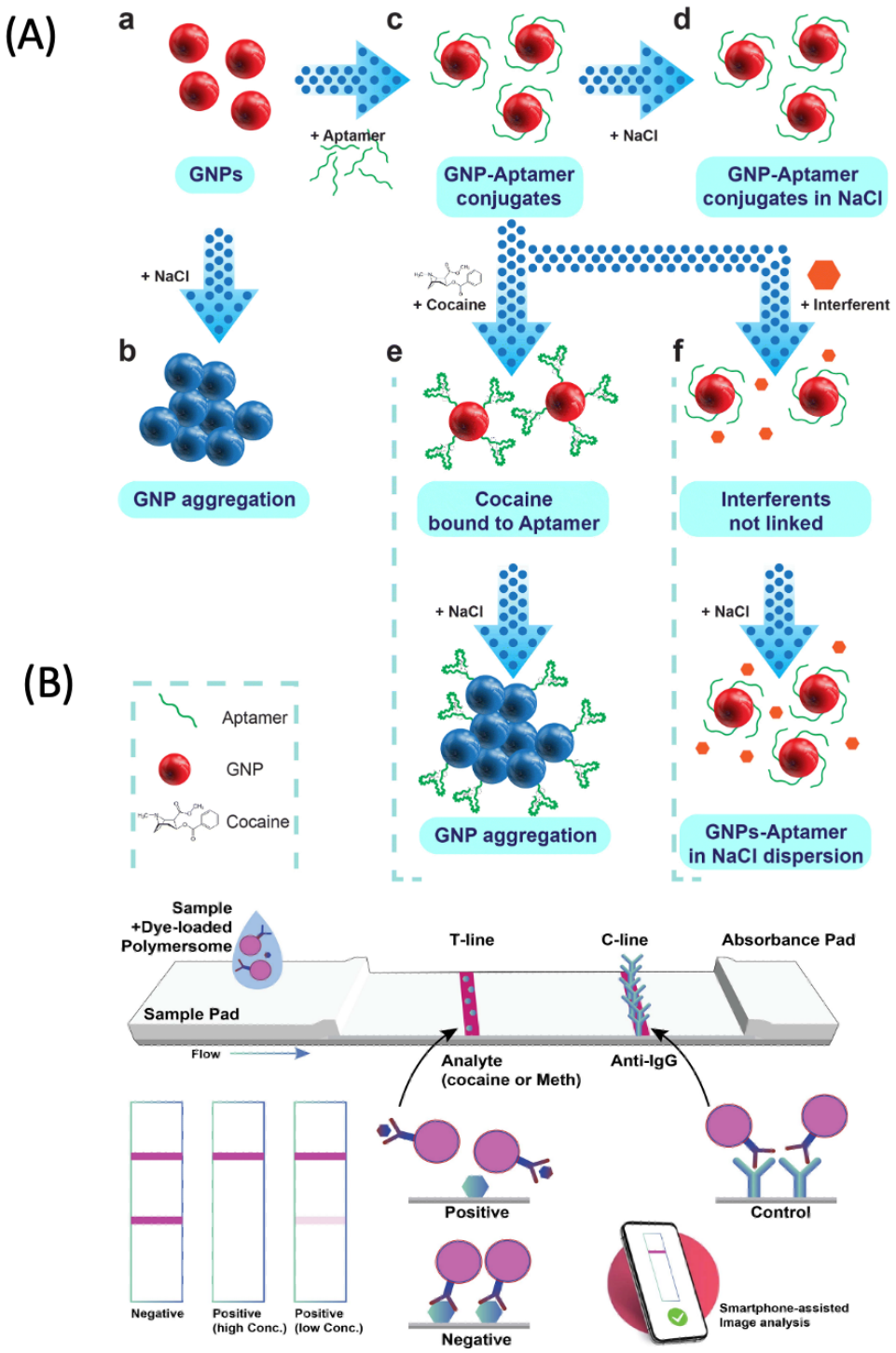

Figure 2-2: (A): Schematic of the mechanism of action of the biosensor. AuNPs aptamers selectively link to COC and change the conformation during an incubation period. The selectivity only allows for this phenomenon to occur with COC and not the interferents)

Reprinted with permission from [18] (B) The bands are observed by the naked eye and later are photographed with a smartphone for quantitative analysis. A positive sample will give a dose-dependent T line colour intensity. Only qualitative results are present © [2022] IEEE [19].

To overcome the limitations seen with colorimetric techniques, electrochemical techniques have been recently adopted. The electrochemical signals that result from the oxidation or reduction of COC offer quantitative results in a matter of minutes [20]. Parrilla et al. [21] recently developed a surfactant-mediated electrochemical sensor using a stripping voltammetry approach. The SPE were functionalized with a surfactant and were proven on COC, heroin, MDMA, and other illicit substances. Although not as lengthy as the colorimetric approaches, this technique required an incubation period of 15 minutes. Some recent reports on the detection of COC include colorimetry with a limit of detection (LoD) of 440 pM (0.15 ng/mL), [22], Square Wave Voltammetry (SWV) with an LoD of 21 nM (7.13 ng/mL) [23], and Differential Pulse Voltammetry (DPV) with and LoD of 28.62 nM (9.72 ng/mL) [24].

2.3.2 Salivary Cocaine Detection Devices Currently in the Market

The collection and use of for drug detection provide an excellent noninvasive alternative. Over the years, the use of for detection has had notable developments; Several devices are currently available for the detection of COC in OF such as the Draeger DrugTest® , Alere DDS2, and IScreen test (Table 2-2). All the current options have several advantages the main one being ease of use. Since the intended users of these devices are police officers and employers, the complexity is an important parameter to optimize. Regarding accuracy and reproducibility, the Draeger DrugTest 500 is the only one

demonstrating promising results [25], [27]. SalivaScreen 5 presents itself as the most cost-effective device and AlereDDS2 and DrugTest 500 can store 10,000 and 500 results respectively.

Table 2-1: Existing COC detection device. Edited table obtained from [28].

Device Name	**Detection Mechanism**	**Testing Matrix**	**Cut-off (ng/mL)**	**Time of Analysis (minutes)**
SalivaScreen 5	Immunoassay Technique	OF	30	10
Draeger DrugTest 5000 Analyzer	Colorimetry	OF	20	<9
Alere DDS2	Lateral Flow Immunoassay	OF	30	<5
IScreen Test	Lateral Flow Chromatography Immunoassay	OF	20	~10
Sali.Check™ System	Immunoassay Technique	OF	20	10-20
Smartclip Multidrug	Immunoassay Technique	OF/ Sweat	20	1

Although they have proven accuracies, no consistent specifications are universally

applied to these devices. Similarly, the cut-off values reported by each company vary since some report the detectability of the drug while others provide the concentration at which the drug can be detected [29]. For the Draeger DrugTest 5000 Analyzer, individuals cannot eat, drink, or smoke within 10 minutes of the test. The same analyzer is also affected by environmental conditions and a negative result does not mean a drug-free sample given that the cut-off levels are not low enough [30]. The same disadvantages are noted with the Alere DDS2, iScreen Test Device, and SalivaScreen 5 [26]. As for other tests, such as Sali.Check-™ System, the factor of time plays a significant role [31].

2.4 Conclusion

Detecting COC and other illicit substances has traditionally been performed using complex and time-consuming equipment. Similarly, the commonly used biological fluids (urine, blood) are collected invasively and at the cost of the individual's privacy. It is known that these methods are highly accurate and effective; however, there is room for improvement, which include tests that require less time, devices that are portable and suitable for roadside applications, and testing in a biological fluid that is less invasive to collect such as OF. This new device should also be accurate, reliable, and withstand varying environmental conditions. The use of electrochemistry to detect COC is becoming increasingly popular, with several devices already on the market being used. Although the devices are effective, several outstanding factors can be improved:

- Testing that takes a few minutes.
- Detecting COC in OF lower than 20 ng/mL.

- Stability through eliminating the use of biomolecule-modified electrodes.

Chapter 3: Sensing in Oral Fluid – Challenges and Perspective

3.1 Preface

This section outlines the properties of oral fluid, collection techniques, and challenges encountered when using oral fluid for the electrochemical detection of analytes. Various sections from this chapter are part of a review paper pending publication. The terms saliva and oral fluid are not used interchangeably in this chapter as they are in the remainder of this thesis.

Ana Gomez Cardoso is the sole author of this section

3.2 Introduction

The testing of biological fluids for disease or drug detection is usually performed using blood or urine [1], [2]. Anything from drug testing to glucose monitoring involves invasive procedures and highly trained personnel to perform the testing and analysis. OF as the primary biological fluid for testing has become increasingly popular. OF is an electroactive fluid that is composed of 99% water. Other components of include saliva, and various electrolytes such as sodium, potassium, proteins, enzymes, and nitrogenous products [3]. The collection of involves a minimally invasive procedure that is also pain-free [4]. Research has shown that analytes found in OF are helpful for disease diagnoses such as cardiovascular irregularities or cancer and forensic medicine, including testing for drug abuse [1], [5].

Although OF-based testing is a promising technology, many scientific reviews shy away from the details involved when working with OF especially in an electrochemical setting. The suitability of testing is seldom explored. Few papers assess the overall impact of diagnostics and most of the time, important information regarding analyte recovery, OF collection, and challenges involved with the overall process is omitted in papers. Although analyte detection in OF has promising advantages, there are several outstanding complications.

- The establishment of correlation between analyte levels in OF when compared to urine and blood
- Food intake and degradation
- Variability between individuals in viscosity and flow rate [5], [7].

Electrochemical sensing with OF as the medium has become increasingly popular for detecting many different analytes [29]. The detection techniques are easy to use, easy to implement, low cost, portable, and ideal for roadside testing [6], [7]. This chapter focuses on the components found in OF and how they interfere with electrochemical testing, the collection for testing as well as recent upgrades made in the field of electrochemistry with OF testing. In parallel, OF testing challenges are explored while providing research-based advancements.

3.3 Oral Fluid Properties

3.3.1 Viscosity and Components

Human OF is a neutral medium ranging from pH 6.0 to 8.0 [4], [8]. OF is a non-Newtonian fluid, making it a complex medium [9]. Non-Newtonian fluids' viscosity can change when under force, and in the case of OF, it also changes based on composition and degradation [3]. As a biological fluid, OF is a mixture of salivary gland excretions from the parotid, sublingual, and mandibular glands (Figure 3-1). Of the major salivary glands, the parotid gland produces the most saliva accounting for over 60% of the total [9]. Saliva is continuously secreted by the glands and is often stimulated by factors such as smells, taste, and chewing [10]. The diversity between the major glands has been studied, and it is of great interest for experiments and real-life applications that involve the use of OF [11]. For example, although the parotid gland delivers the most saliva during chewing, it delivers the least during passive periods [12]. Alternatively, the submandibular gland releases saliva under the tongue which indicates the most flow during periods of rest [13].

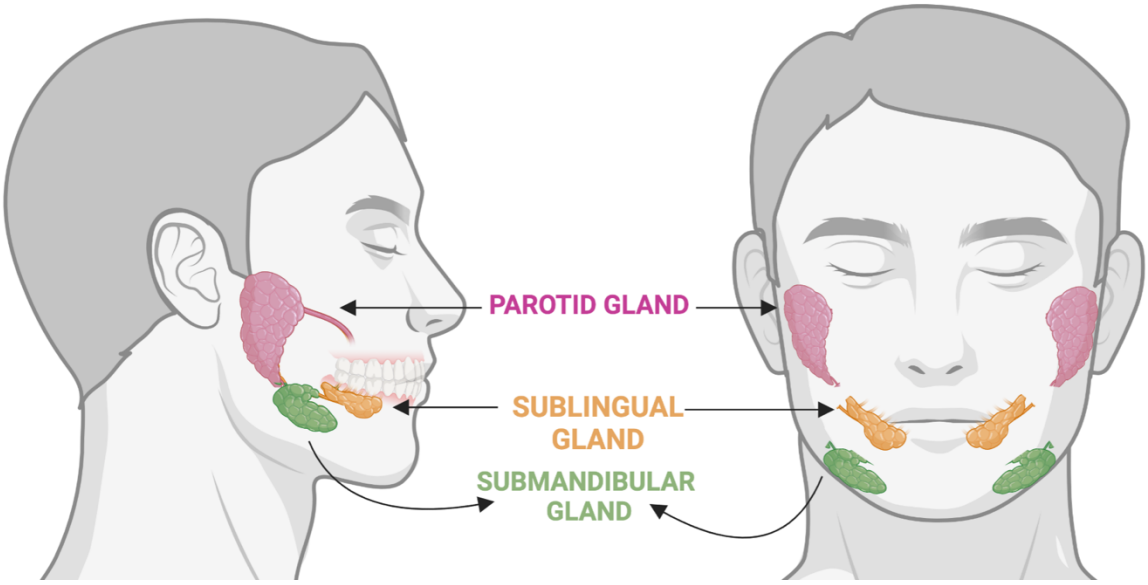

Figure 3-1:Salivary glands in human mouth.

The two main cell types involved in the production of saliva are the acini cells and the ductal cells [14]. The acini cells produce and secrete fluid containing electrolytes, mucus, enzymes, and water. This OF then flows to the ductal cells which modify and deliver the OF to one's mouth [9], [11], [15]. OF has many components including primary proteins, mucins, enzymes, and water [16]. The mode of action of these primary components is outlined in Table 3-1. OF is a primary component in maintaining oral health. It protects teeth from microbes and facilitates articulation as well as mastication and digestion [17]. The effects and differences between passive OF collection and stimulated OF collection will be discussed in this paper as it pertains to electrochemical experiments and results. Furthermore, diseases, diet, and medication all influence OF excretion and viscosity [18].

Table 3-1: Primary components in OF in addition to their purpose and modes of action

Component	Purpose/Function	Mode of Action	References
Mucins	• Oral lubrication • Antimicrobial functions	• Aids and promotes swallowing • Transporting substances to taste buds	[7], [10], [19]–[21]
Enzymes	• Initial digestion • Mastication	• Initial digestion of food • Antimicrobial, antifungal, and antiviral • Provide nutrition for certain internal microbiota	[7], [10], [19]–[21]
Water	• Articulation/speech • Oral lubrication	• Remove microorganisms • Dilute foods	[7], [10], [19]–[21]

	• Oral clearance	• OF texture	
Proteins	• Antimicrobial functions • Oral hygiene	• Important for tooth health and inhibiting calcium precipitation	[7], [10], [19]–[21]

3.4 Oral Fluid Collection

3.4.1 Collection by External Stimulation

Passive OF production excludes any external stimulating factors and allows for simply the OF that accumulates in one's mouth to be collected. External factors such as smells, or food stimulate the saliva glands to produce more OF than passive production. Research from Malik et al. [22] and Jenkins et al. [20] demonstrated that OF sometimes has less of the analyte when compared to blood or urine; therefore, external stimulation is often required.

Collectors such as Salivette or lab-made dupes often use citric acid to stimulate OF production [23]–[25]. The external stimulation increases OF production up to an order of magnitude when compared to unstimulated production [16]. Although it is often necessary to stimulate OF production, the use of citric acid has proven to be controversial [36], [37]. Citric acid stimulation changes the pH of saliva and in turn alters the concentration of the drug in OF [7], [38]. Research conducted by O'Neal et al. [29] concluded that concentrations of codeine in OF were higher in samples obtained by spitting than those obtained by acidic stimulation. Another factor that plays an important role in stimulated OF collection is flow rate. As seen in Table 3-2, most of the research performed with expectoration as the collection method is paired with a commercial collection device whereas passive drooling (the desired method of collection) can use any type of collector.

Table 3-2: Different analytes and the respective saliva collection methods

Collection Method	**Collection Device**	**Analyte**	**Electrochemical Technique**	**Ref**
Passive drool	Eppendorf tube	Uric acid	Chronoamperometry	[30]
Passive drool/Stimulated	Eppendorf tube	Glucose	Amperometry	[31]
Expectoration	Salivette	THC/ethanol	SWV	[32]
Passive drool	Eppendorf tube	Glucose/lactate	CV	[33]
Expectoration	Eppendorf tube	Caffeine	Potentiometric	[34]
Passive drool	Eppendorf tube	Ascorbic acid	Chronoamperometry	[35]
Stimulated with collection device	Salimetrics/ Salivette/ Salivette Cortisol	Cortisol	SWV	[6]
Expectoration	Orasure	COC	SWV	[36]
Passive drooling	Eppendorf tube	Lactate	CV	[37]
Passive drooling	Eppendorf tube	Glucose	CV	[38]
Expectoration	Glass tubes	Cadmium/Lead	SWV	[39]
Expectoration	Eppendorf tube	THC	SWV	[40]
Stimulated (Cotton swab)	Salivette	Methadone	CV	[41]
Stimulated (Cotton swab)	Salivette	Cotinine	DPV/CV	[42]
Passive drooling / Expectoration	Glass vial	Cortisol	DPV/CV	[43]

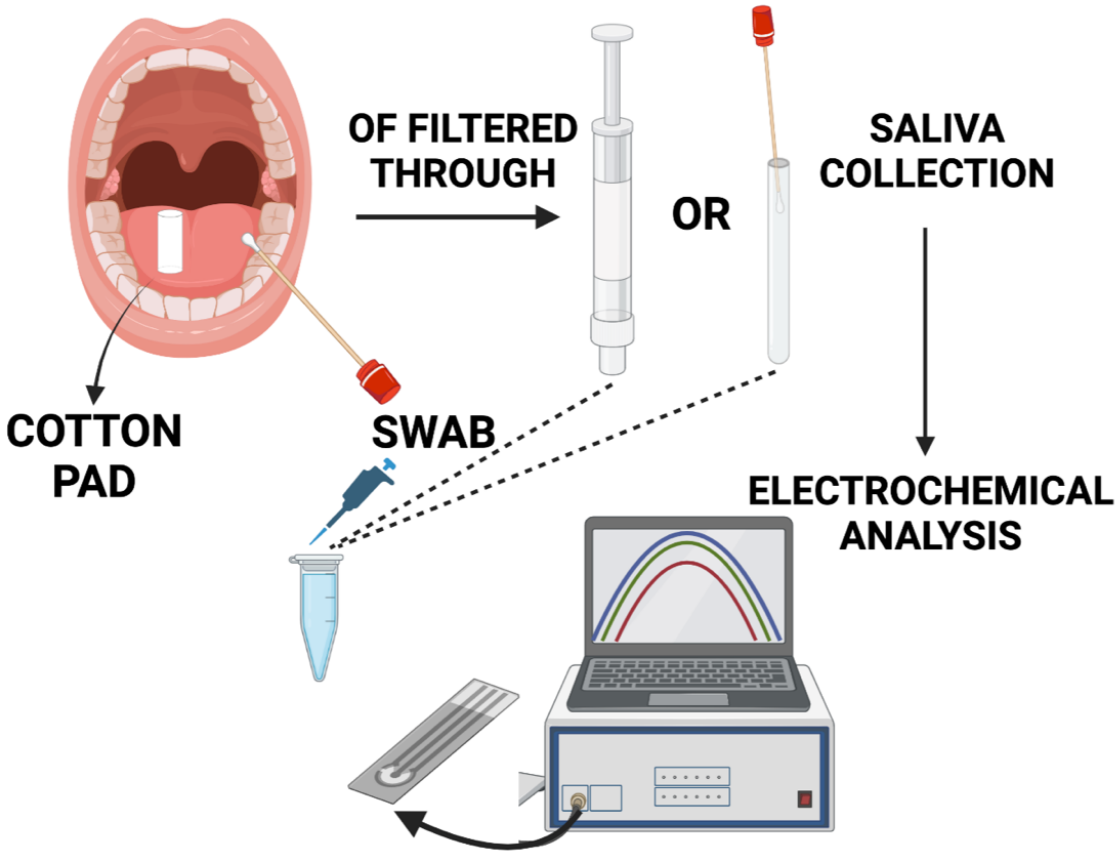

Figure 3-2: Saliva collection procedure from using swabs or cotton pads directly to electrochemical analysis.

Another method of stimulated collection is implemented using swabs. The swabs can be placed inside an individual's mouth and the material used for swabbing can vary and highly depends on the tolerability of the subject (Figure 3-2). The material used also has a large impact on the retention of the analyte recovery [44]. Another advantage of stimulated collection is that researchers can avoid the collection of whole saliva samples. By placing the cotton swab in specific parts of the mouth, specific saliva can be collected (i.e., sublingual, parotid, and submandibular) Figure 3-1 [11].

3.4.2 Passive Drooling

The passive drooling of OF involves unstimulated OF to "deburr" (letting the OF drop) into the collection tube. Passive drooling has been used since the 1930s and is the most optimal method of collection because the flow rate parameter is negligible. The advantages of passive drooling have been highlighted by Hodgson el al. [45] who mentioned the effect of ignoring salivary flow. Although this method is considered the best, it is not accessible to certain individuals such as young children and senior citizens [45]. Also, the homogeneity of passive OF samples is not guaranteed [30]. There are devices in the market specifically designed for passive drooling [16], [46] such as the Saliva Collection Aid by Salimetrics [47]. This device is used after the subject has pooled OF in their mouth and collection proceeds by tilting the person's head forwards towards the collection vial. Similarly, Proflow Sialometer ™ by Proflow Incorporated manufactured a device for the collection of passive drools which is composed of a funnel attached to the collection vial as well as the individual's lower lip [16]. Kamarainen et al. [6] developed a disposable sensor for the electrochemical detection of cortisol in human OF and employed a passive OF collection method. The authors opted for passive collection to use the same saliva sample pool. Passive collection using the Salivette Cortisol ™ collector avoids the inclusion of flow rate as one of the parameters.

Oasis Diagnostic has developed two devices that complement the collection of unstimulated OF. The SuperSal and VersiSal are also used for other biological fluids such as urine and amniotic fluid [48]. The collectors work by placing an absorbent pad into the individual's mouth and allowing saliva to pool while simultaneously being absorbed. After

collection (roughly 1.2 mL in 2 minutes), the absorbent pad is placed into a compression tube and the OF is squeezed into an Eppendorf tube [48], [49]. Ikemoto et al. [50] reported an electrochemical sensor for the detection of salivary uric acid. The saliva was collected in two different methods: One being passive drool and the other by absorption from the participant's sublingual glands. The passive drool collection tool used was the commonly used Salimetrics tool. The overall conclusion of this research was that in the samples collected with passive drool, the levels of uric acid were higher than the samples that used saliva from sublingual glands. This example demonstrates the differences between stimulated and unstimulated collection and the effects it can have on overall results. However, this conclusion was contradicted by Hernandez et al. [18] who analyzed the influence of sampling conditions for measuring uric acid in OF. The main difference between the research performed by Hernandez et al. [18] and Ikemoto et al. [50] was the type of collector used (Salimetrics collector for Ikemoto et al. and Salivette for Hernandez et al.). Perhaps a more solid conclusion can be made if both researchers had used the same collection device under the same conditions.

3.5 Analyte Recovery

The collection of OF is not standardized and the variation in materials used to obtain the OF result in a drastic difference in the overall recovery of the desired analyte. Analyte recovery testing is an important step to evaluate when measuring different concentrations whether electrochemically or using other techniques. The overall result of a recovery analysis depends on what type of OF was used i.e., saliva produced by specific glands or

whole saliva, as well as the material of the collector [49]. Although analyte recovery could introduce a lot of variability in the results, it is one of the least explored sides of salivomics. Mishra et al. [32] developed an electrochemical sensor for the simultaneous detection of Δ^9-tetrahydrocannabinol (THC) and ethanol using a wearable ring sensor. The team used a Salivette collection device which uses centrifugation to collect the OF that has been absorbed into the cotton pad. Although the group did not perform an analyte recovery, Teixeira et al. [51] did in their THC detection in OF analysis. While the authors did not detect THC electrochemically, the recovery experiments are isolated and very important in the overall scope of any THC detection project. Following the Salivette instructions, the group reported that THC could not be detected 65% of the time. During 40 separate trials, 26 samples that were spiked with THC were undetectable after undergoing collection with the Salivette device. The alternative which is direct expectoration was preferred even though the Salivette device is still a great methodology for OF collection [51].

Table 3-3: Summary of COC recovery with different collectors [52].

	Control	**Salivette**	**Intercept**	**Finger Collector**	**ORALscreen**	**Hooded Collector**
5 ng/mL		4.23	4.69	3.02	3.41	3.23
		4.42	4.37	3.06	3.47	3.22
	4.73	4.3	4.37	2.99	3.26	3.34
	4.62	4.22	4.62	3.05	3.31	3.26
	4.68	4.33	4.28	3.11	3.33	3.06
Mean	0.08	4.30	4.47	3.05	3.36	3.22
SD		0.08	0.18	0.05	0.08	0.10

% Control		91.98	95.53	65.16%	71.79	68.92%
10 ng/mL		8.11	8.54	5.32	6.08	5.84
	8.87	8.13	8.07	5.18	5.57	6.27
	8.85	7.85	8.88	5.55	5.94	6.26
	8.86	7.96	8.4	5.74	6.5	6.67
	0.01	8.51	9	5.57	6.04	5.46
Mean		8.11	8.58	5.47	6.03	6.10
SD		0.25	0.37	0.22	0.33	0.46
% Control		91.26%	96.82%	61.76%	68.01%	68.85%
100 ng/mL	86.95	75.57	76.95	50	55.81	55.39
	84.08	77.99	84.02	51.63	58.88	51.78
	85.52	77.4	83.47	56.8	58.56	54.78
	2.03	78.29	81.71	52.25	56.6	52.51
		79.18	80.98	50.28	59.97	51.99
Mean		77.69	81.43	52.19	57.96	53.29
SD		1.35	2.79	2.74	1.71	1.67
% Control		90.84%	95.22%	61.03%	67.78%	62.32%

Quintela et al. [53] explored the recovery of various drugs after collection with the Quintesal ™ Oral Fluid Collection Device and other competing collectors such as the

Salivette Collector. The analytes tested included THC, morphine, COC, amphetamines, and other commonly abused illicit substances. After recovery testing, the group reported that when using the Quantisal collector, the recovery of amphetamines was at least 93% while using the other collectors, the recovery percentage did not exceed 59% [53]. Though recovery was acceptable for amphetamines, the results for THC were very poor and not reported at all in the paper. Lomonaco et al. [54] researched the recovery of lactate, and uric acid using three different Salivette collection devices including one with a cotton swab, a cotton swab with citric acid and polyester swabs. The team also explored the effects of pH during collection. However, the range of pH was small (pH 5.3-8.1). In the results shown, pH did not make a difference when detecting either analyte since both had a collection average of higher than 96% [54]. One of the other analytes the group was researching was chloride. However, in this case, the recovery analysis was deemed unnecessary given that chloride is an ionic compound. Besides pH, the temperature of the saliva is an important parameter to investigate given that Bilancio et al. [28] reported that the levels of uric acid in saliva fluctuated depending on its freshness. Table 3-3 outlines several other collectors and the corresponding recovery percentages.

3.6 Challenges and Barriers

3.6.1 Salivary Interferences

Although the use of OF for analyte detection has many advantages, there are negative factors that also need to be considered. Bacteria quickly degrade OF samples [55]. Furthermore, OF is likely to contain food, leftover debris, and traces of legal drugs that

can affect the detection of the desired analyte [56]. Matrix effects refer to other components in a testing sample besides the desired analyte. During the chemical analysis, the matrix can drastically impact the outcome and quality of the results. As previously discussed, the collection methods make a significant difference in the detection analysis. Furthermore, it has been proven that the pH of saliva is affected by the flow rate, which also affects the analytes' excretion [4], [52]. This effect was demonstrated by Malik et al. [22] during stimulated saliva collection and passive saliva collection. The results concluded that stimulated collection increased the pH of saliva and resulted in lower analyte concentration.

Since OF is an electroactive medium, various components often interfere with the analyte of interest during electrochemical analysis. Mishra et al. [32] developed an electrochemical ring sensor for the simultaneous salivary detection of ethanol and THC. The researchers reported no interference from the OF matrix. The study reports the use of the whole saliva which indicates that it was not diluted or altered in any sense. Alternatively, Ortega et al. [40] reported salivary interference when analyzing the electrochemical detection of THC on screen-printed electrodes. The group reports the composition of different simulated OF cocktails that contained common organic components found in saliva such as uric acid, glutamate, and ascorbic acid. The group stated that uric acid was the main interference due to its electrochemical oxidation at 0.4 V. Certain proteins in saliva including uric acid and ascorbic acid also oxidized within the 0.4 to 0.6V potential range which makes it difficult to strictly analyze the analyte of choice [40].

3.6.2 Blood/Urine/Saliva Correlation

Establishing a correlation between OF and blood levels depends on the specific analyte, and it is often difficult to do. Blood is the standard biological fluid when testing for diseases or even the presence of illicit substances. Years of research dedicated to studying the pharmacokinetic effects of markers and substances in blood have provided hundreds of guidelines that are backed with many experiments to prove their validity [57]. Establishing a correlation between the biological fluids is challenging and not all analytes necessarily show a correlation. Those analytes that do are still subject to discrepancies due to OF interferences such as flow rate and food intake [5]. Despite the challenges, a lot of progress has been made regarding the correlation of biological fluids while using electrochemical detection methods. In general, studies have demonstrated that OF mainly contains the parent drug. For example, in the case of COC, though it is rapidly metabolized in the liver, the major analyte present in OF is COC itself [58]. A similar concept is seen after smoking cannabis where THC is the predominant analyte found in OF [25].

Schepers et al. [59] researched the pharmacokinetics of amphetamines in OF with eight voluntary participants. The drug was detectable in OF within 0.08-2h with the initial concentrations ranging from 24.7-312.2 ng/mL. The peak concentrations were found from 2-12 hours with an average of 14.3 ng/mL. The plasma/OF correlation was analyzed and given that the drug dose was administered orally, it was found that the concentration was higher in OF than in plasma. The plasma/OF correlation was inconsistent and varied widely between participants. The plasma/OF ratios varied from 0-24. Overall, the conclusion was a poor correlation between plasma and OF that was confirmed with a low R^2 value of 0.222

[59].

In a more recent study where a therapeutic drug monitoring approach was implemented, Whokittel et al. [60] researched the correlation between amphetamine concentrations in OF and in serum. The results obtained in this study were very similar to those from Schepers et al. [59]. The concentration of amphetamine was higher in OF than in serum and the serum/OF ratio was highly variable and dependent on salivary pH with an overall conclusion that OF is not an appropriate matrix for the detection of amphetamines [60]. A lot of effort has also been made for the detection of blood glucose from salivary testing [2], [61], [62]. Malik et al. [22] examined a non-invasive platform for the estimation of blood glucose levels from salivary parameters. The results demonstrated an R^2 value of 0.45 which indicates a very weak correlation between blood and salivary glucose levels [22], [63]**.** On the other hand, Wang et al. [38] found an excellent correlation between OF and blood levels of glucose utilizing glassy carbon electrodes with nanorods ($R^2 = 0.99$) In their work, the authors found that the OF collected was stimulated with glucose while Malik et al. [22] used a combination of expectoration and passive drooling. As stated, the method of collection can have a drastic effect on overall results.

Fiorentin et al. [64] conducted research in which the concentration of COC was analyzed in various biological fluids. After administration of COC by smoking, OF, blood, and urine were obtained from 64 individuals. While the mean concentration (in ng/mL) was 46.88 in OF, the concentration in plasma was 10.68 and 713.65 in urine [64]. Significant correlations were obtained between OF and plasma ($R^2 = 0.17$) and OF and urine ($R^2 = 0.07$). One of the main points of this research is that the mode of administration (intranasal,

intravenous, smoked) has a significant impact on the concentrations found in OF, blood, and urine [64].

3.7 Conclusion

Electrochemical sensing in OF has become increasingly popular due to the noninvasive collection of OF paired with the accurate and quantitative results obtained when using electrochemistry. Moderate research has been done regarding the use of OF as an alternative to blood and urine using other techniques however, the challenges and obstacles faced when using electrochemistry are underreported or not reported at all. Although convenient, sensing in OF still requires a lot more research to be a feasible alternative.

3.8 References

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Chapter 4: Electrochemical Detection of Cocaine in PBS

4.1 Preface

This chapter focuses on the development of a biomolecule free sensor for the detection of COC. Starting by identifying the oxidation potential of COC on carbon electrodes, this chapter outlines the detailed optimization procedure to develop a chemical sensor that was proven to detect nanoscale concentration of COC in PBS. The results from this chapter are pending patent applications. The contributors to this chapter include:

Ms. Ana Gomez Cardoso

Dr. Syed Rahin Ahmed – Part of **4.3.3 Gold Nanoparticle Synthesis**

Dr. Herlys Viltres – **4.4.4.1 XPS Characterization and part of Figure 4-2 analysis**

4.2 Introduction

According to Driving Under the Influence of Drugs, Alcohol and Medicines (DRUID) and the Roadside Testing Assessment (ROSITA) project, COC detection devices should not only have a success rate of over 80%, but it should also ideally take a few minutes for the overall analysis [1], [2]. Though progress has been made, recent techniques do not demonstrate a method for detecting COC under five minutes without incubation periods that can detect low concentrations of the drug in oral fluid.

To address the various limitations of existing devices, a novel electrochemical sensing approach has been developed to detect COC in phosphate buffer solution (PBS). At first, the WE of the SPE was modified with the same analyte being detected (COC). The affinity of the modified SPE surface towards the addition of different concentrated COC samples

allows for very low-level detection of COC in PBS to be performed in less than one minute.

4.3 Experimental Details

4.3.1 Materials and Equipment

Electrodes were rinsed with ultrapure (Milli-Q water), and solutions were prepared using PBS purchased from Sigma Aldrich as tablets. Cocaine hydrochloride, THC, Chloroauric acid ($HAuCl_4.3H_2O$), sodium borohydride ($NaBH_4$) and Cysteamine hydrochloride were all purchased from Sigma-Aldrich, USA. ELISA Cocaine Oral Fluid Kit was purchased from Neogen Corporation. Fresh stock solutions were prepared when performing each experiment to achieve the most accurate results.

The electrochemical experiments were performed using a PalmSens 4 Potentiostat (Basi, Bioanalytical Systems Inc., USA) connected to a computer using the PalmSens PSTrace Software. SPE with carbon-based working (3 mm/0.071 cm^2) and CE and silver RE were purchased from Zensor R&D, Taiwan. The X-ray photoelectron spectroscopy (XPS) analyses were carried out with a Kratos AXIS Supra X-ray photoelectron spectrometer using a monochromatic Al K(alpha) source (15mA, 15kV). XPS can detect all elements except hydrogen and helium, probes the surface of the sample to a depth of 7 - 10 nanometers, and has detection limits ranging from 0.1 - 0.5 atomic percent depending on the element. The instrument work function was calibrated to give a binding energy (BE) of 83.96 eV for the Au 4f7/2 line for metallic gold, and the spectrometer dispersion was adjusted to give a BE of 932.62 eV for the Cu 2p3/2 line of metallic copper. The Kratos charge neutralizer system was used on all specimens. Survey scan analyses were carried

out with an analysis area of 300 x 700 microns and a pass energy of 160 eV. High-resolution analyses were carried out with an analysis area of 300 x 700 microns and a pass energy of 20 eV. Spectra were charge-corrected to the C-C, C-H line of the carbon 1s spectrum (aliphatic carbon) set to 285.00 eV. Spectra were analyzed using CasaXPS software (version 2.3.14). Finally, data analysis and image configuration were performed using the Origin 8.5 software (OriginLab, United States).

4.3.2 Modification of Screen Printed Electrodes Using Cocaine

The carbon electrodes used were thoroughly rinsed with Milli-Q water and airdried prior to starting the experiments. As a pre-treatment, 100 µL of PBS were dispensed on the electrode and interrogated under the following SWV parameters: equilibration time of 3 s, voltammetric potential scan from 0 to 1.5 V with a frequency of 15 Hz, an amplitude of 25 mV, and a step potential of 5 mV. The same SWV parameters were used for the identification of the COC oxidation peak. This pre-treatment step was repeated three times per electrode. Next, a solution composed of PBS and methanol (MetOH) (9:1 ratio in volume) was prepared to be mixed with the COC as the modifying solvent. The PBS-MetOH solution was mixed with COC at a 9:1 ratio in volume, referred to as the COCi solution. This solution was then used to obtain an initial COCi deposition of various amounts of COC depending on how much was dispensed on the WE (Table 4-1). For 150 ng of COC, 1.5 µL of the solution was drop-casted on the WE. After dispensing, the solution was airdried for approximately six minutes on the WE.

Table 4-1: COCi Solution Preparation

COCi Amount (ng)	Amount of PBS:MetOH (9:1): Amount of COC (1mg/mL) (µL)	Amount Dispensed on Electrode (µL)
100	36:4	1
150	36:4	1.5
200	36:4	2
500	20:20	1
1000	-	1

4.3.3 Cocaine Attachment to Working Electrode

A modified version of an ELISA method was used to confirm this attachment. Initially, several electrodes were modified using the standard COCi solution while a control group was left un-modified. Then, AuNPs conjugated with COC binding antibody solution were pipetted on the WE after the COCi solution was absorbed and kept for one hour at room temperature. Following another washing step with PBS, 3,3',5,5'-Tetramethylbenzidine (TMB) was pipetted on the electrode, and to stop the reactions, hydrogen peroxide (10%) was deposited on the electrode. To obtain a reading of the results, the solution on the electrodes was pipetted onto a 96-well plate. The readings were done using a UV-visible spectrometer (Synergy H1, BioTek, Winooski, VT, USA) at a wavelength of 450nm.

4.3.3.1 Gold Nanoparticle Synthesis

The (+) AuNPs were prepared using the published protocol [3]. A cysteamine solution (400 µL, 213 mM) was added to 40 mL of 1.42 mM $HAuCl_4$ solution. The solution was stirred for 20 minutes at room temperature. After stirring, ten µL of 10 mM $NaBH_4$ solution was added, and the mixture was stirred for 25 min at room temperature in the dark. The synthesized AuNPs were stored in the fridge at 4°C for further use. The AuNPs were characterized with UV-Visible absorption spectra and transmission electron microscopy (TEM). The results of TEM showed the size of the AuNPs was about 34 nm.

Table 4-2: Sample preparation for COC in PBS

Dilution Obtained From:	**Concentration of COC in Fluid (ng/mL)**	**Volume of fluid (µL)**	**Volume of COC (µL)**
	0	900	-
1000 ng/mL	10	990	10
Serial dilution 1000-500-250 ng/mL	25	500	500
Serial dilution 1000-500-250 ng/mL	50	500	500
10,000 ng/mL	100	990	10
Serial dilution 1000-500-250 ng/mL	250	500	500
Serial dilution 1000-500-250 ng/mL	500	500	500
10,000 ng/mL	1000	900	100

1 mg/mL	10,000	990	10

4.3.4 Cocaine Detection in PBS

Once the electrodes were modified with COCi, they were used for COC detection in PBS. The samples were prepared using a serial dilution method of COC in PBS ranging from 0 to 1000 ng/mL. (Table 4-2); 100 µL of the COC spiked PBS samples were individually pipetted on the electrode to cover the entire area. The parameters of CV were as follows: equilibration time of 5 s, voltammetric potential scan from 0 to 1.5 V, E step of 0.01 V/s, and a scan rate of 0.1 V/s.

4.4 Results and Discussion

4.4.1 Bare Screen-Printed Electrode Performance

According to several sources, the oxidation of COC on graphite SPE occurs in the potential range of 1 to 1.1 V [4]–[6]. The first step toward the detection of COC on the Zensor electrodes was to identify the oxidation peak. Therefore, high concentrations of COC dissolved in PBS were interrogated using SWV. Concentrations from 0 to 50,000 ng/mL of COC in PBS were prepared via serial dilution. High concentrations of COC in PBS demonstrated sharp, well-defined peaks at around 1-1.1V (Figure 4-1).

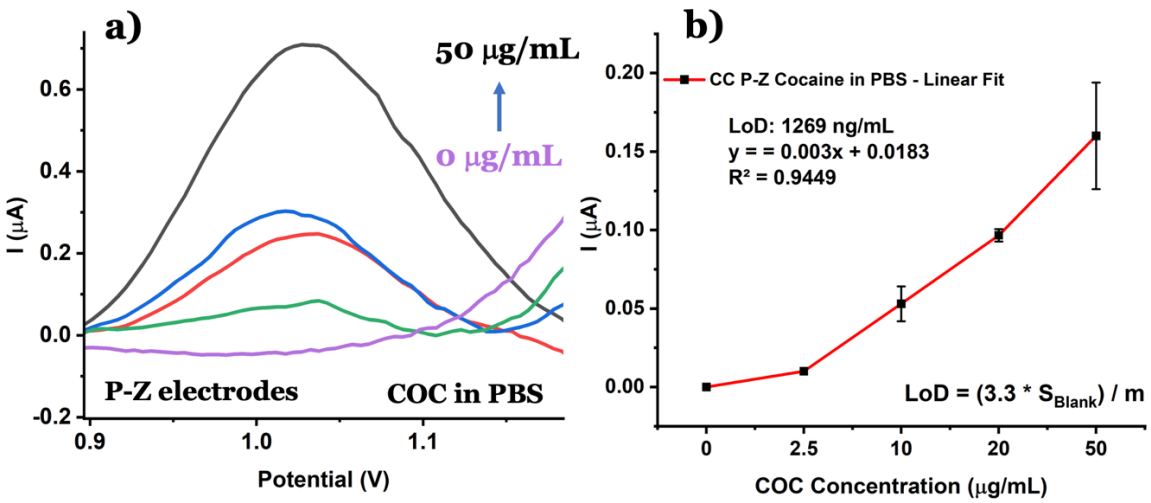

Figure 4-1 (a) SWV signals of different amounts of COC and (b) calibration curve for COC detection on bare electrodes (P-Z) using PBS.

As the concentration decreased, the current intensity became visibly weaker, and on P-Z, any concentration lower than 2500 ng/mL of COC in PBS did not develop a visible signal. On a P-Z, the lowest concentration that can be detected is $LoD_{bareelectrode}$ = 1269 ng/mL (Figure 4-1), which does not comply with any of the cut-offs set by drug safety enforcement organizations [5], [6]. The completion of this analysis further proved the need for modification of the electrode.

4.4.2 Modified Sensor Performance

The electrode modification includes an approach that uses the same analyte that is being detected to modify the electrode. The primary purpose of modifying the electrodes is to increase the selectivity and sensitivity toward the desired analyte. Previous reports by Ortega et al. [7] and Renaud et al. [8] demonstrate the use of electrodes being modified by using the same analyte during the manufacturing as linker points to facilitate the interaction

between the samples between interrogated and the electrode. In this case, the electrodes are modified (m-Z-COC) by depositing COCi on the WE, which is absorbed on the surface of the WE. This methodology is traditionally accompanied by electrochemical interrogation to immobilize the analyte on the WE [7]. However, during experimental research, it was established that once COC oxidizes on the electrode, there are no adduct molecules remaining. The adduct molecules that remain in the work of Renaud et al. [8] and Ortega et al. [7] facilitate the further oxidation of other molecules found in the samples. Since all the COC molecules oxidize under CV interrogation, a different approach was implemented. The proposed explanation for improving the detection is enhanced physical interactions and chemical coupling. The physical adsorption preserves the activity and the stability of the COC analyte [9]. To further analyze this interaction, electrochemical characterization, as well as XPS analysis, was performed. To the best of the author's knowledge, this approach has not been previously implemented or manufactured for the electrochemical detection of COC. However, similar work has been attempted to detect THC [7], [8].

4.4.3 Sensor Optimization in PBS

Two different approaches were evaluated to implement the COC deposition on the electrode WE. The first approach included the preparation of a COC solution in a mix of H_2O: MetOH (9:1) before deposition (m-Z-COC*) (Figure 4-2c, I). In the second case, COC was dispersed in a mix of PBS:MetOH (9:1) (Figure 4-2c, II). XPS and CV techniques were implemented to explain the interaction of COC with the WE surface in both conditions (I and II).

Two peaks were employed to fit the N 1s high-resolution signal after COC:H_2O/MetOH deposition on the WE (m-Z-COC*) (Figure 4-2 a). For this specific case, m-Z-COC*, the atomic percent of the second contribution corresponding to the protonated amine (43.1%) was higher than the one obtained for the m-Z-COC when COC/PBS:MetOH is employed for deposition (Figure 4-2b). The presence of a higher number of protonated amine (condition I) on the WE does not allow the oxidation of the tertiary amine groups on the COCi structure, avoiding further detection of the COC analyte (Figure 4-2c and e). Meanwhile, in the deposition based on PBS:MetOH, the buffer fixes a local pH on the WE, allowing the deprotonation of the COCi amine, favouring the tertiary amine oxidation (Figure 4-2 d and e). On the same note, the pH of deionized water ranges from 6 to 6.4, whereas the pH of the PBS solution used was 7.4, which is closer to the pKa of COC at 8.6.

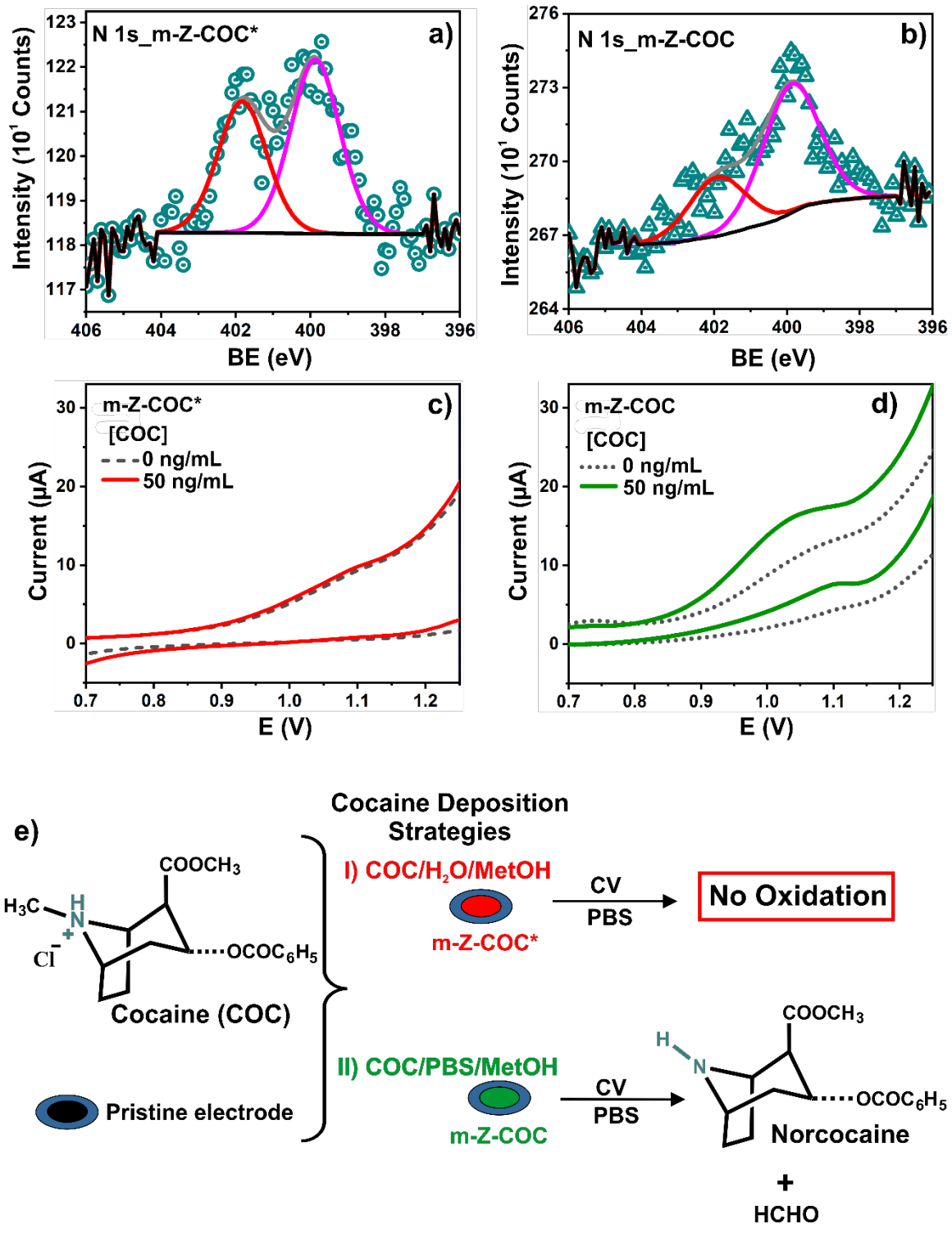

Figure 4-2: a) and b) N 1s high-resolution spectra of m-Z-COC and m-Z-COC. c) and d) COC detection using m-Z-COC* and m-Z-COC electrodes employing CV. e) Schematic representation of the mechanism for COC electrochemical oxidation.*

One of the first parameters to be optimized was the amount of COCi that would be used to modify the WE. Figure 4-3 b demonstrates that when the electrode is not modified with the COCi solution, there is no signal present; however, the m-Z-COC electrode provides a distinguishable signal between 0 and 50 ng/mL . To optimize the signal and avoid electrode saturation, varying amounts of COCi were used to modify the electrode (100, 150, 200 and 500 ng of COCi).

To follow the traditional electrodeposition technique, immediately after dispensing COCi on the WE, the electrodes would be interrogated with PBS under SWV parameters to immobilize the COC. However, in this case, the COC was fully oxidized on the first scan (Figure 4-3 b). After an initial scan, there was no COC left which would result in a loss of amperometric amplitude. The electrodeposition step was eliminated from the protocol and replaced with an absorption deposition and increased signal strength to overcome this issue. Figure 4-3 c shows that using a low amount such as 100ng COCi does not provide accurate results. After testing the 75 ng/mL of COC in PBS sample, the intensity decreases for 100 ng/mL when in theory it should increase since there is more COC present. Nonetheless, with just 50 ng of COCi more, 150ng COCi provides excellent results that easily distinguish between 0 and 50 ng/mL. Not only that, but the intensity for 100 ng/mL of COC in PBS is higher (Figure 4-3c).

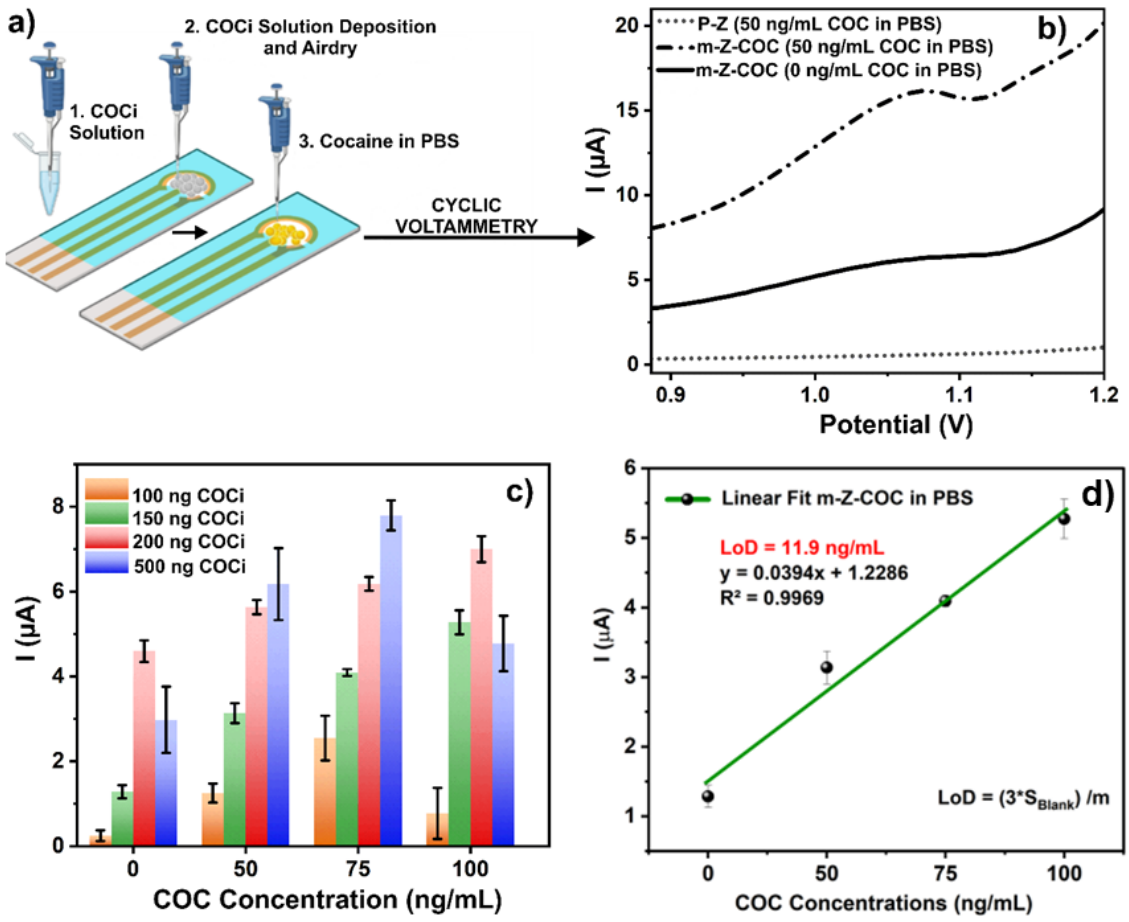

Figure 4-3: a) Schematic of electrode modification. b) Detection of COC (50 ng/mL) using P-Z and modified electrodes (m-Z-COC) in PBS. (c) Optimization of COCi amount to modify the electrode, and d) COC detection in PBS using COCi 150ng.

Several airflow conditions were tested for drying the electrodes after modification. Initially, using cool airflow for 30 seconds allowed the electrodes to dry quickly; however, the signal intensity decreased compared to other methods. Hot airflow for 20 seconds was also analyzed (Figure 4-4 a). While the signal had higher intensity than cool airflow, the error bar doubled in size, indicating that the hot airflow interferes with the COC deposition. A combination of the methods was also attempted; however, allowing the electrodes to

airdry produced the optimal results.

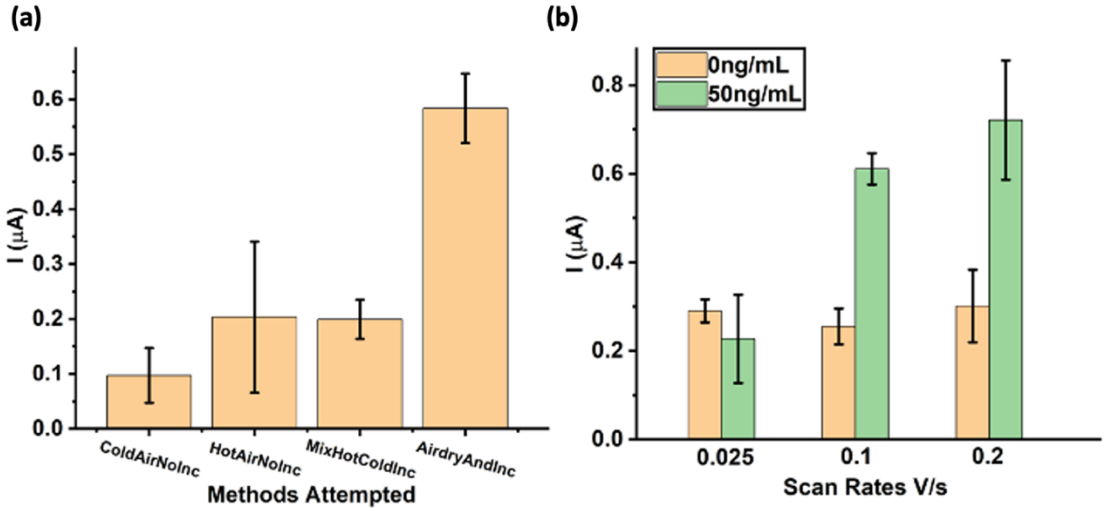

Figure 4-4: (a) Standard deviation analysis of optimization experiments for drying electrodes after deposition (b) Standard deviation analysis of different scan rates using CV.

The scan rate parameter of CV was also optimized since it also affects the intensity of the peak alongside the optimization to dry the COCi solution on the WE. An ongoing drawback of the detection was the broad peaks, which directly indicate the electron transfer occurring during oxidation. Using CV as the new electrochemistry technique would introduce changing the scan rate. Higher scan rates also produce sharper peaks with higher intensity (Figure 4-4 b). Three different scan rates were analyzed: 0.025, 0.1, and 0.2 V/s. As predicted, the lowest scan rate (0.025 V/s) resulted in the weakest signal, whereas the highest scan rate (0.2 V/s) resulted in the strongest signal. Ultimately, as shown in Figure 4-3, the scan rate of 0.1 V/s provided the optimal results with an increase in current intensity and a decrease in the error bars. It was determined that the LoD of COC in PBS was 11.9 ng/mL (Figure 4-4 d), which is well below the official cut-off.

4.4.4 Electrode Characterization

4.4.4.1 XPS

Once the electrode modification was optimized, several characterization experiments took place. XPS characterization technique was employed to study the surface of the SPE before and after COC modification due to all chemical processes in the electrode modification step occurring on the surface of the WE. The XPS survey profiles confirmed the presence of C, O, N, S, P, and Si elements in all the samples under study (Table 4-3).

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Table 4-3: XPS survey data (atomic percentage) for the most concentrated elements for the materials

Samples	**Elements (At. %)**									
	C 1s	**O 1s**	**N 1s**	**Cl 2p**	**S 2p**	**P 2p**	**Si 2p**	**Fe 2p**	**Na 1s**	**K 2s**
P-Z	82.2	8.7	0.4	7.6	0.3	0.1	0.8	-	-	-
P-Z-PBS	78.6	9.0	0.1	10.4	0.2	0.4	0.6	0.1	0.7	-
m-Z-COC*	81.1	8.8	0.4	8.6	0.2	0.3	0.2	0.2	0.1	-
m-Z-COC	66.5	8.3	0.3	16.3	0.1	0.8	0.1	0.1	7.1	0.4

m-Z-COC1	76.6	9.8	0.3	10.7	0.1	0.6	0.5	0.2	1.2	-

Fe, Na, and K elements were evident in the survey's spectra after the P-Z cleaning with PBS using CV and COC modification. Also, the atomic percentage of Cl increased after COC modification (m-Z-COC) (Figure 4-5 a). Additionally, a decrease in the atomic percentage of N was observed after cleaning with PBS (m-Z-PBS), but this percentage increased with the COC modification because nitrogen (N) is one of the elements present in the COC structure added to the WE (Figure 4-5 a, Table 4-3). The C 1s high-resolution signal of the cleaned P-Z could be deconvoluted into four contributions at 284.3, 285.0, 286.4, and 288.9 eV attributed to C=C aromatic, C-C/C-H, C-OH/C-O-C/C-Cl, and O-C=O, respectively (Figure 4-5 b, Table 4-5). After COC modification, four signals were also employed to fit the C 1s signals for the rest of the samples (m-Z-COC, m-Z-COC1, and m-Z-COC*). However, in the specific case of m-Z-COC, a decrease in the first contribution (aromatic C=C) is observed, which could be related to fewer C=C functional groups in the COC structure than in the graphite-based ink of the WE.

Table 4-4: The peak-fitting results of C 1s high-resolution signal of materials.

Samples	**Assignment**	**E_B (eV)**	**FWHM (eV)**	**At. %**
P-Z	**C1s** C=C aromatic	284.4	0.6	16.2
	C1s C-C, C-H	285.0	1.3	57.6
	C1s COH, C-O-C, C-Cl	286.5	1.3	22.0
	C1s O-C=O	289.0	1.3	4.2

P-Z-PBS	**C1s** C=C aromatic	284.3	0.7	21.3
	C1s C-C, C-H	285.0	1.3	49.4
	C1s COH, C-O-C, C-Cl	286.4	1.3	25.2
	C1s O-C=O	288.9	1.5	4.1
m-Z-COC*	**C1s** C=C aromatic	284.4	0.7	19.1
	C1s C-C, C-H	285.0	1.3	50.8
	C1s COH, C-O-C, C-Cl	286.4	1.3	25.4
	C1s O-C=O	288.7	1.5	4.7
m-Z-COC	**C1s** C=C aromatic	284.5	0.6	11.8
	C1s C-C, C-H	285.0	1.3	52.1
	C1s COH, C-O-C, C-Cl	286.3	1.4	32.0
	C1s O-C=O	288.8	1.5	4.1
m-Z-COC1	**C1s** C=C aromatic	284.3	0.7	22.4
	C1s C-C, C-H	285.0	1.2	48.9
	C1s COH, C-O-C, C-Cl	286.4	1.2	24.8
	C1s O-C=O	288..8	1.4	4.0

Table 4-5: The peak-fitting results of O 1s high-resolution signal of materials.

Samples	**Assignment**	**E_B (eV)**	**FWHM (eV)**	**At. %**
P-Z	**O1s** C=O	532.5	1.5	68.3
	O1s O*-(C=O)-C, C-O aromatic	533.5	1.6	31.7

P-PBS	**O1s** O-C=O	532.0	1.7	59.0
	O1s O*-(C=O)-C, C-O aromatic	533.4	1.7	41.0
	O1s Na Auger	536.2	1.7	0.0
m-Z-COC*	**O1s** O-C=O	532.0	1.7	60.9
	O1s O*-(C=O)-C, C-O aromatic	533.4	1.7	39.1
	O1s Na Auger	536.1	1.8	0.0
m-Z-COC	**O1s** O-C=O	532.0	1.7	57.3
	O1s O*-(C=O)-C, C-O aromatic	533.5	1.7	42.7
	O1s Na Auger	536.5	2.1	0.0
m-Z-COC1	**O1s** O-C=O	532.0	1.7	55.7
	O1s O*-(C=O)-C, C-Oaromatic	533.4	1.7	44.3

The high-resolution signal of N 1s was deconvoluted into two contributions for all the samples (P-Z, P-Z-PBS, m-Z-COC, m-Z-COC1, and m-Z-COC*) (Table 4-6). In the P-Z after PBS cleaning using CV (m-Z-PBS), the first contribution at 399.9 eV was related to amine/amide from the ink of the WE [10]. The second contribution at higher binding energies (401.9 eV) is attributed to protonated amines [11]. (Figure 4-5 d, Table 4-6). After COC electrode modification, slight changes are observed in both contributions. The peak related to amine/amide increased (1.6%) due to the presence of the tertiary amine group in the COC structure incorporated on the WE surface (Figure 4-5 d, Table 4-6). However, when the COC-based electrode is interrogated using CV in PBS, significant changes are observed in both peaks of N 1s high-resolution signal (Figure 4-5 f, Table 4-6).

Table 4-6: The peak-fitting results of N 1s high-resolution signal of materials.

Samples	Assignment	E_B (eV)	FWHM (eV)	At. %
P-Z	**N1s** Amine, Amide	399.6	1.8	58.1
	N1s $_{NR4+}$	401.9	1.8	41.9
P-Z-PBS	**N1s** Amine, Amide	399.9	1.5	67.0
	N1s $_{NR4+}$	401.9	1.5	33.0
m-Z-COC*	**N1s** Amine, Amide	399.9	1.5	56.9
	N1s $_{NR4+}$	401.8	1.6	43.1
m-Z-COC	**N1s** Amine, Amide	399.9	1.8	68.6
	N1s $_{NR4+}$	401.9	1.8	31.4
m-Z-COC1	**N1s** Amine, Amide	400.0	1.9	84.9
	N1s $_{NR4+}$	402.3	1.9	15.1

The atomic percent of the first contribution related to amine functional groups increased from 68.4 to 84.9; meanwhile, the percent of the second contribution (protonated amines) decreased from 31.4 to 15.1 (Table 4-6). This result confirms the oxidation of the tertiary amine group present in the COC structure. In the oxidation of this amine, the abstraction of an electron from the amino–nitrogen is carried out, followed by a proton loss to form a neutral radical. The neutral radical loses an electron and is hydrolyzed to the products. The oxidation reaction leads to a secondary amine, norcocaine, and an aldehyde [4] (Figure 4-5 e). Therefore, XPS corroborated that the amine group plays the main role in the electrochemical oxidation of COC molecules on the surface of the WE.

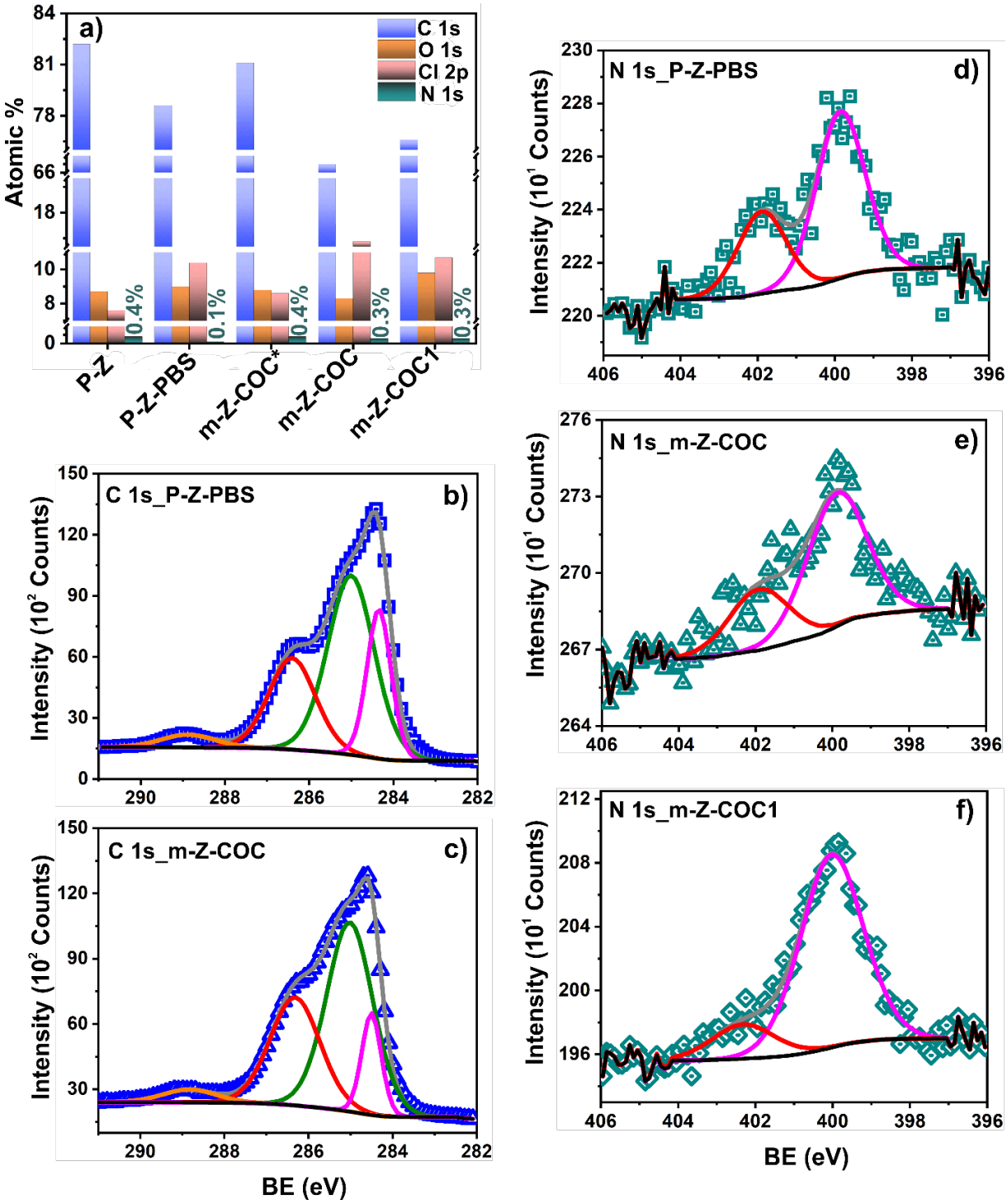

Figure 4-5: a) Element quantification from survey spectra; b), c), C 1s and d), e), f) N 1s high-resolution spectra of P-Z and m-Z-COC.

4.4.4.2 Electrochemical Characterization

Electrochemical changes after modifying the electrodes with the COCi solution were studied using a standard redox probe of $K_4[FeCN_6]$ (FeCN) 0.1 mM in KCl 0.1 M [9].The carbon-based SPE purchased from Zensor presented a geometric area of 0.07 cm^2 and its electrochemically active working area of 0.05 cm^2, rendering it 71% effective (Figures 4-6 a and b). When modifying the WE with the COCi method, the electrochemically active working area was 31% effective (Figure 4-6 c). This was calculated using the Randles-Sevcik equation.

Although the electroactive area of the electrode decreased, it was demonstrated through other methods of characterization that the affinity towards the target analyte increased. From this electrochemical analysis, it was also established that the oxidation of COC is an irreversible reaction. As seen in Figure 4-6 b, the peaks of the voltammogram are close, indicating the reversibility of the $K_4[FeCN_6]$ (FeCN) 0.1 mM in KCl 0.1 M reaction on a Z-P. However, Figures 4-6 c and d exhibit the voltammograms of electrodes that have been modified with COCi. The distance between each peak is greater than 60mV, indicating an irreversible reaction [12].

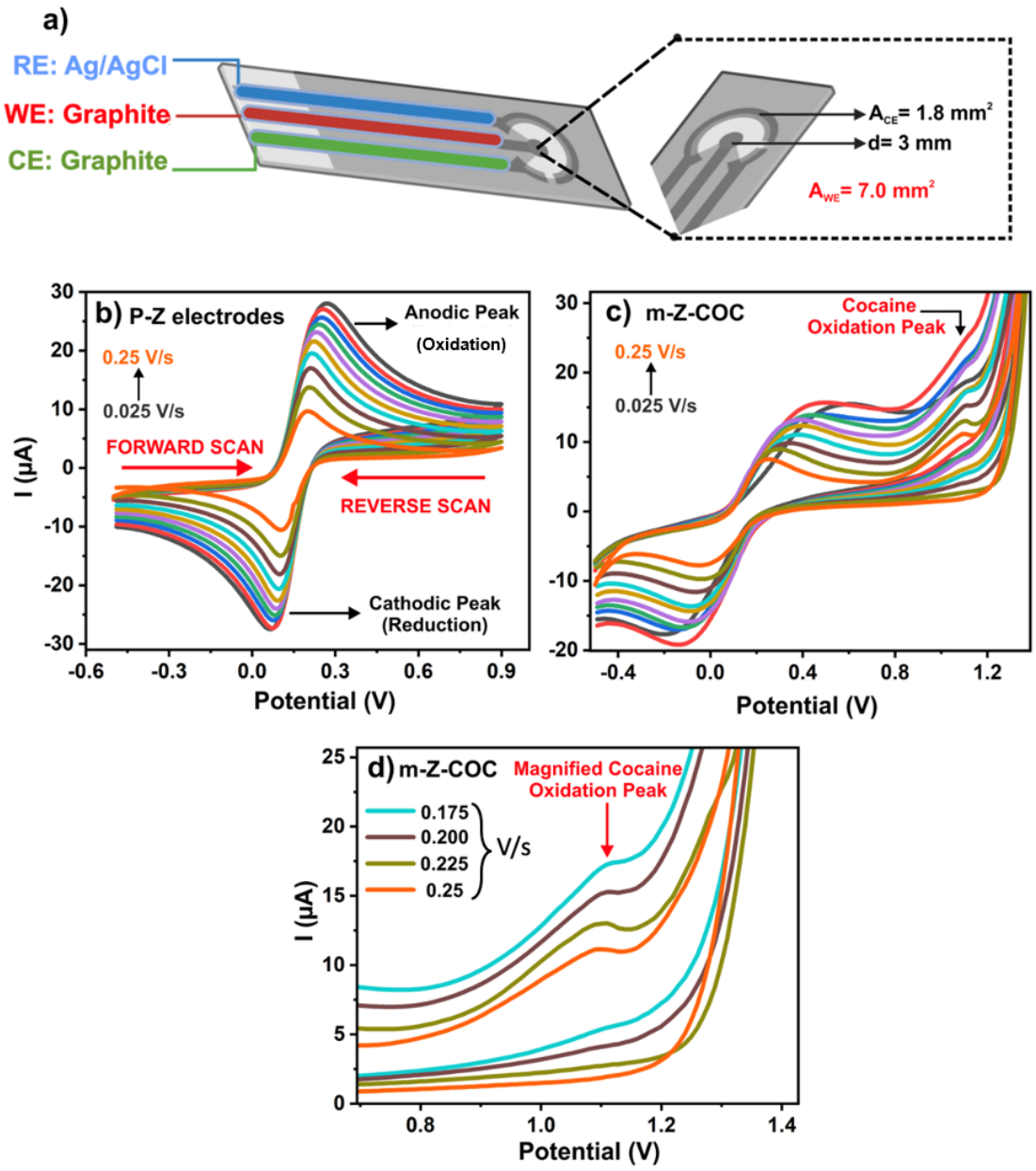

Figure 4-6: (a) Configuration of a Zensor electrode. (b) CV response of a P-Z with different scan rates. (c) CV response of m-Z-COC with different scan rates. (d) Magnified COC peak in m-Z-COC scans.

Figure 4-6 b shows the scans of P-Z, while Figure 4-6 c represents COC peaks at different scan rates when m-z-COC is employed. In the magnified image (Figure 4-6 d),

for higher scan rates, 0.175 V/s and above, the peaks are sharper and more defined, whereas lower scan rates develop broad and weak peaks. This information is prevalent and provides a better understanding of how COC molecules act in an electrochemical setting. To further analyze the kinetics of the COC oxidation of a carbon electrode, CV was used with various scan rates from 0.025 to 0.2 mV/s using 50 ng/mL of COC in PBS. Given that the current increased linearly with the square root of the scan rate, it can be concluded that the COC oxidation follows a diffusion-controlled process which is supported by Mirceski et al. [13]

4.4.4.3 Verification of Cocaine Attachment to Working Electrode

The electrodes were further characterized by analyzing the COC attachment to the carbon SPE using a modified ELISA. To evaluate the attachment of COC molecules on the WE, a modified ELISA method was performed where the entire process was performed on the WEs. COC was deposited onto the WE in the same way that electrodes are modified, and after waiting for the COC solution to be absorbed, a COC binding antibody solution was pipetted onto the electrode.

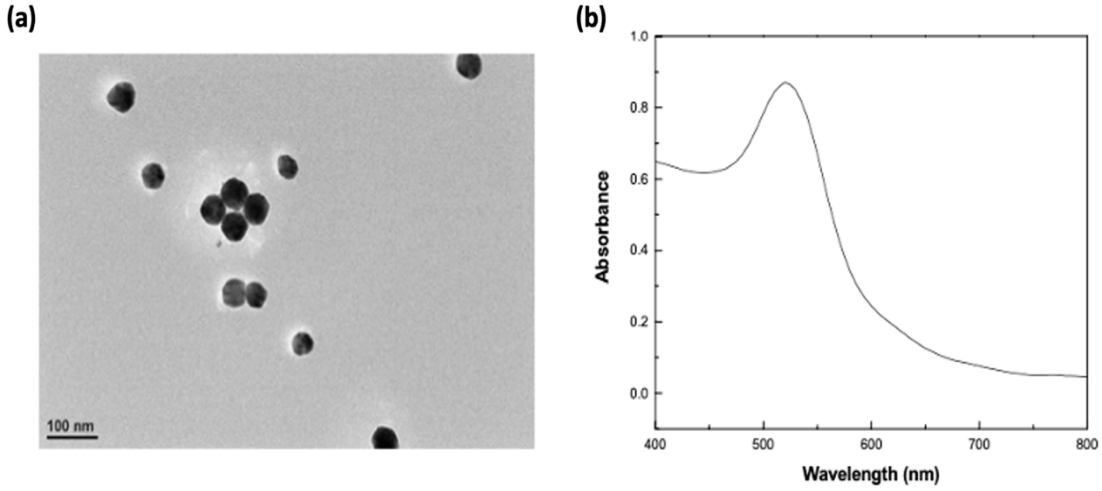

Figure 4-7: (a) TEM of the prepared AuNPs. (b) UV-vis absorption spectra of the prepared AuNPS

Normally, this step proceeds with depositing HRP as the secondary antibody to bind to the primary antibody used; however, this step was altered, and AuNPs were used instead. The use of nanoparticles has become increasingly popular due to their wide range of applications. AuNPs are easy to use, synthesize, and functionalize, which makes them a powerful tool in many applications. A prevalent application is the use of functionalized AuNPs for antigen detection [14]. Van der Heide et al. [14] recently used functionalized AuNPs for the detection of COC while exploring several methods of functionalization. Similarly, Gao et al. [15] used a similar approach for the detection of COC with a LoD 10 mM. Therefore, the use of AuNPs for COC detection is becoming a more conventional method that is not only easier but also more cost-efficient.

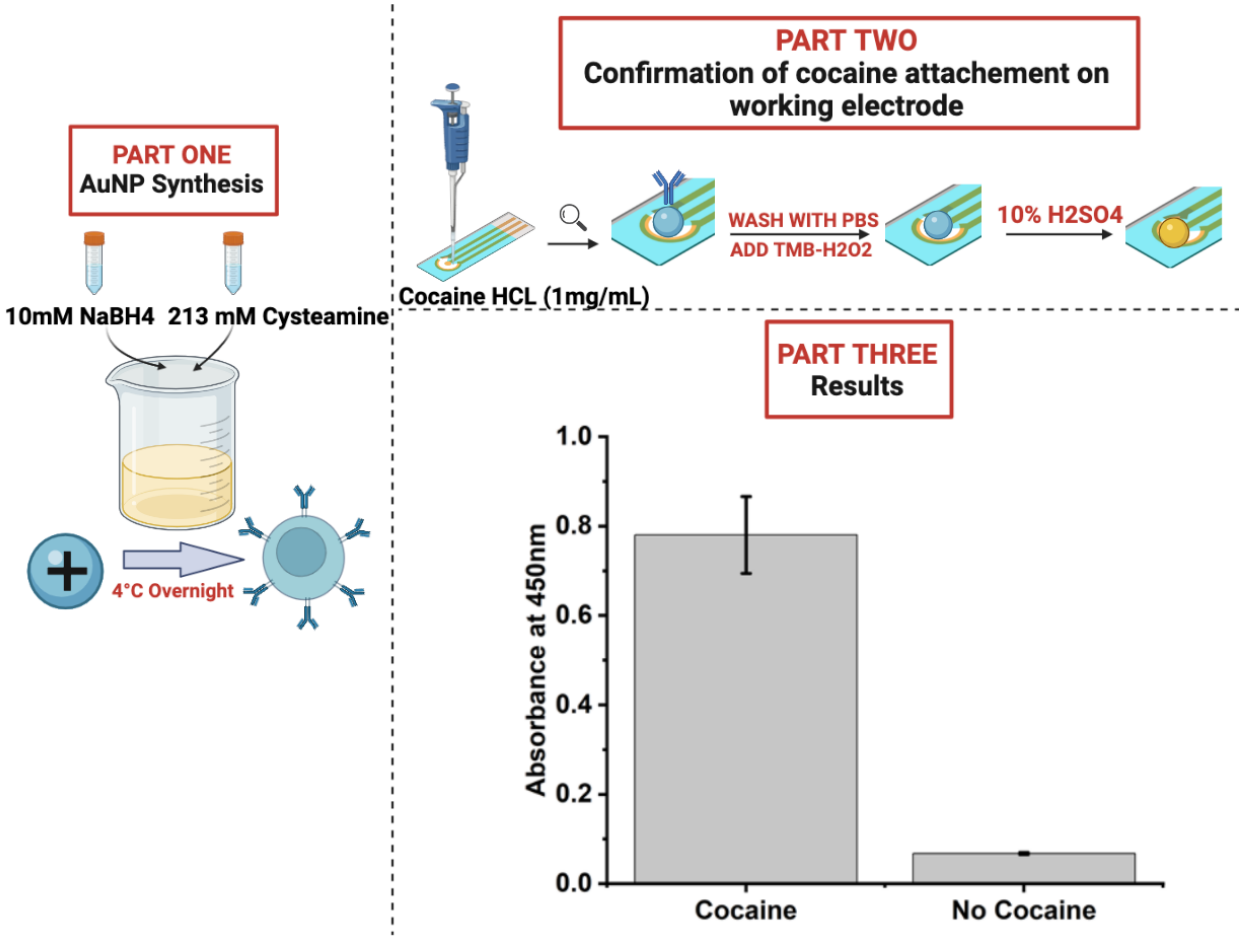

Figure 4-8: Part 1: Au NP synthesis. Part 2: Experimental diagram of AuNPs being deposited on the WE. Part 3: Results of experiments.

The results revealed that the absorbance intensity obtained from the COC-modified electrodes (m-Z-COC) is much higher than the control, which indicates the successful attachment of COC with the WEs (Figure 4-8). Moreover, it can be concluded that the attachment of COC on the WEs is strong and consistent even after several washing steps.

4.5 Conclusions

It was established that P-Z are unable to detect concentrations of COC in PBS below

1269 ng/mL. To proceed and develop a chemical sensor that could detect nanoscale concentrations of COC, several optimization steps were undertaken. One of the main optimization parameters was the amount of modifying solution to be drop cast on the WE. By attempting different amounts and proceeding with other optimization experiments, such as the method of deposition, it was determined that the optimal amount of COCi was 150 ng. Similarly, the traditional use of electrodeposition for the modification solution was eliminated from the protocol since the COC would oxidize fully, not leaving COC on the WE to increase affinity. The next logical step for this application was to detect spiked samples of COC in human saliva, which is explored in Chapter 5.

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Chapter 5: Sensitive Electrochemical Detection of Cocaine in Oral Fluid

5.1 Preface

This chapter focuses on using the data obtained in chapter four but using human saliva as the biological testing matrix of choice. This chapter covers the difficulties encountered with oral fluid and the implementation of machine learning to overcome the challenges. The results from this chapter are pending patent applications The contributors of this chapter include:

Ms. Ana Gomez Cardoso

Dr. Hoda Mozaffari – Machine Learning Algorithms

Dr. Herlys Viltres – Assistance with figure configuration

5.2 Introduction

One of the major barriers in commercializing sensors is challenges relating to the accuracy and reliability. Experiments performed in a laboratory setting under near-perfect conditions are difficult to replicate in the field. Machine learning (ML) is a discipline that is becoming increasingly popular in a broad range of applications. Chemometrics has been widely accepted in analytical chemistry and is one of the most useful approaches to overcoming the challenges of electrochemical sensors [1], [2]. ML algorithms address well-known issues in sensing such as saliva interference and noisy signals. By training ML models, the analyte signal can be easily distinguished from the signal noise. In chapter 4,

the detection of COC in PBS was successfully demonstrated however, the detection of the drug in saliva proved to be more complicated. Manual calculations were not sufficient and other challenges addressed in chapter 3 became evident. By implementing the knowledge from the detection of COC in PBS in chapter 4 and introducing ML as the primary analysis method, the detection of COC in saliva was possible achieving an accuracy in results of 85%.

Other confirmatory experiments were analyzed in this chapter one of which is *hook effect* analysis. A common obstacle when working with electrochemical sensors is the *hook* effect that can occur at high or low concentrations [3]. The term hook effect refers to the flat or parabolic shapes of the signal response. When a low-dose hook effect is present, samples containing low analyte concentrations produce uncharacteristic high results. On the other hand, a high-dose hook effect results in high concentrations producing signals that correspond to low concentrations [4]. The analysis of the hook effect is critical since the wrongful determination of an individual's COC concentration in their saliva can have personal and societal consequences. Lastly, experiments with other drugs and common cutting agents of street COC were performed to ensure the selectivity of the sensor towards COC.

5.3 Experimental

5.3.1 Materials and Equipment

Electrodes were rinsed with ultrapure Milli-Q water, and solutions were prepared using PBS purchased from Sigma Aldrich as tablets. Cocaine hydrochloride, (−)-trans-Δ^9-

THC, ethanol (85%), levamisole hydrochloride, and caffeine in MetOH were all purchased from Sigma-Aldrich, USA. ELISA Cocaine Oral Fluid Kit was purchased from Neogen Corporation. Fresh stock solutions were prepared when performing each experiment to achieve the most accurate results. The electrochemical experiments were performed using a PalmSens 4 Potentiostat (Basi, Bioanalytical Systems Inc., USA) connected to a computer using the PalmSens PSTrace Software. SPE with carbon-based working (3 mm/0.071 cm^2) and counter electrodes and silver reference were purchased from Zensor R&D, Taiwan. Data analysis and image configuration were performed using the Origin 8.5 software (OriginLab, United States).

5.3.2 Saliva Collection

The fresh saliva sample was collected from healthy lab members and used to prepare a series of COC dilutions (0, 10, 25, 50, 100, 250, 500, and 1000 ng/mL) in microtubes. Then, an absorbent pad was placed inside the microtube to collect the different concentrated COC samples separately. This pad was then introduced into a syringe (3 mL) containing a glass wool filter. The absorbed pad was squeezed with a plunger, and filtered samples were collected into a new microtube. The recovered COC after filtration was analyzed using an ELISA Cocaine oral fluid kit.

5.3.3 Cocaine Detection in Saliva

Once the electrodes were modified with COCi, they were used for COC detection in saliva. The samples were prepared using a serial dilution method of COC in saliva ranging

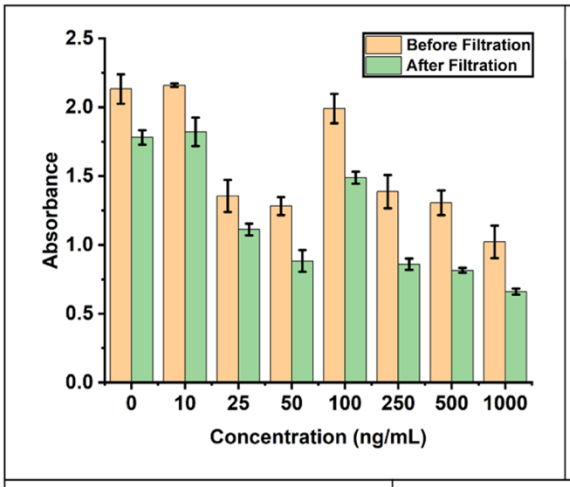
from 0 to 1000 ng/mL. (Table 4-2); 65 µL of the COC spiked saliva samples were individually pipetted on the electrode to cover the entire area. The parameters of CV were as follows: equilibration time of 5 s, voltammetric potential scan from 0.4 to 1.9 V, E step of 0.01 V/s, and a scan rate of 0.1 V/s.

5.4 Results and Discussion

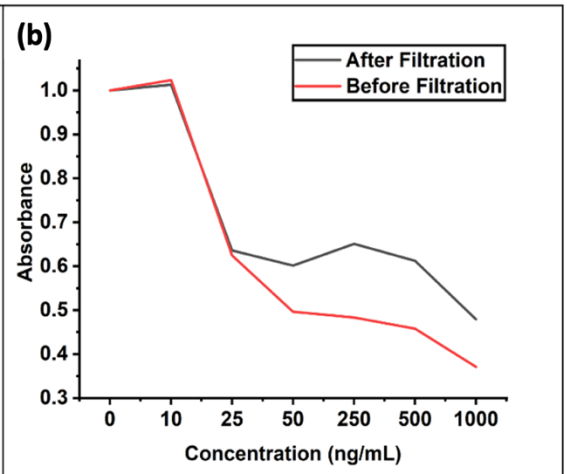
5.4.1 COC Detection in Saliva

After optimizing the detection of COC in PBS and understanding the electrochemical performance of the m-Z-COC, the next logical step was to assess the method in human saliva. Before proceeding with the detection, the recovery of COC in saliva after filtration was analyzed. An ELISA test was performed to evaluate samples of COC in saliva before and after filtration (Figure 5-1). As expected, there was some COC loss after filtering the spiked saliva; however, as shown in Figure 5-1, the loss of COC at concentrations below 100ngmL^{-1} is not significant, managing to remain above 80% recovery. The loss is slightly higher for the higher concentrations (250, 500, 1000 ng/mL) with a recovery percentage of 74-77%. These results show that the filtering device does not hinder the results and the detection of COC in saliva.

(a)



(b)



(c)

Concentration	After	Before	%
0	1	1	100%
10	1.013133	1.023408	101%
25	0.63571	0.624532	98%
50	0.601626	0.496255	82%
100	0.933709	0.835768	90%
250	0.650563	0.483146	74%
500	0.612414	0.457865	75%
1000	0.479362	0.371161	77%

Figure 5-1: (a) (b) Absorbance comparison of samples before and after filtration. (c) Percent recovery for each concentration tested in saliva after filtration.

Pertaining to the detection of COC in saliva, after CV interrogation, at first glance, there was no visible peak for COC in the expected potential region of 1-1.1 V (Figure 5-2 a). The only visible peak was a large and sharp peak at around 1.4 V, which could be attributed to one or a few of the electroactive components found in saliva. To obtain a visible signal, a subtraction of the curves method was implemented to overcome the imminent challenge. While m-Z-COC were scanned with specific concentrations of COC in saliva, a P-Z was also scanned with the same spiked samples. This arrangement is like those found in electronic tongues (ET), where an array of sensors works together to identify

a specific analyte [2]. It has been established that a P-Z cannot detect low concentrations of COC either in PBS or in saliva. Hence, the signal obtained from the P-Z scanned with a spiked sample was representative of the electroactive interferents found in saliva. By subtracting the data obtained from the P-Z from the signal received from an m-Z-COC, the signal demonstrates the correlation between current intensity and analyte concentration.

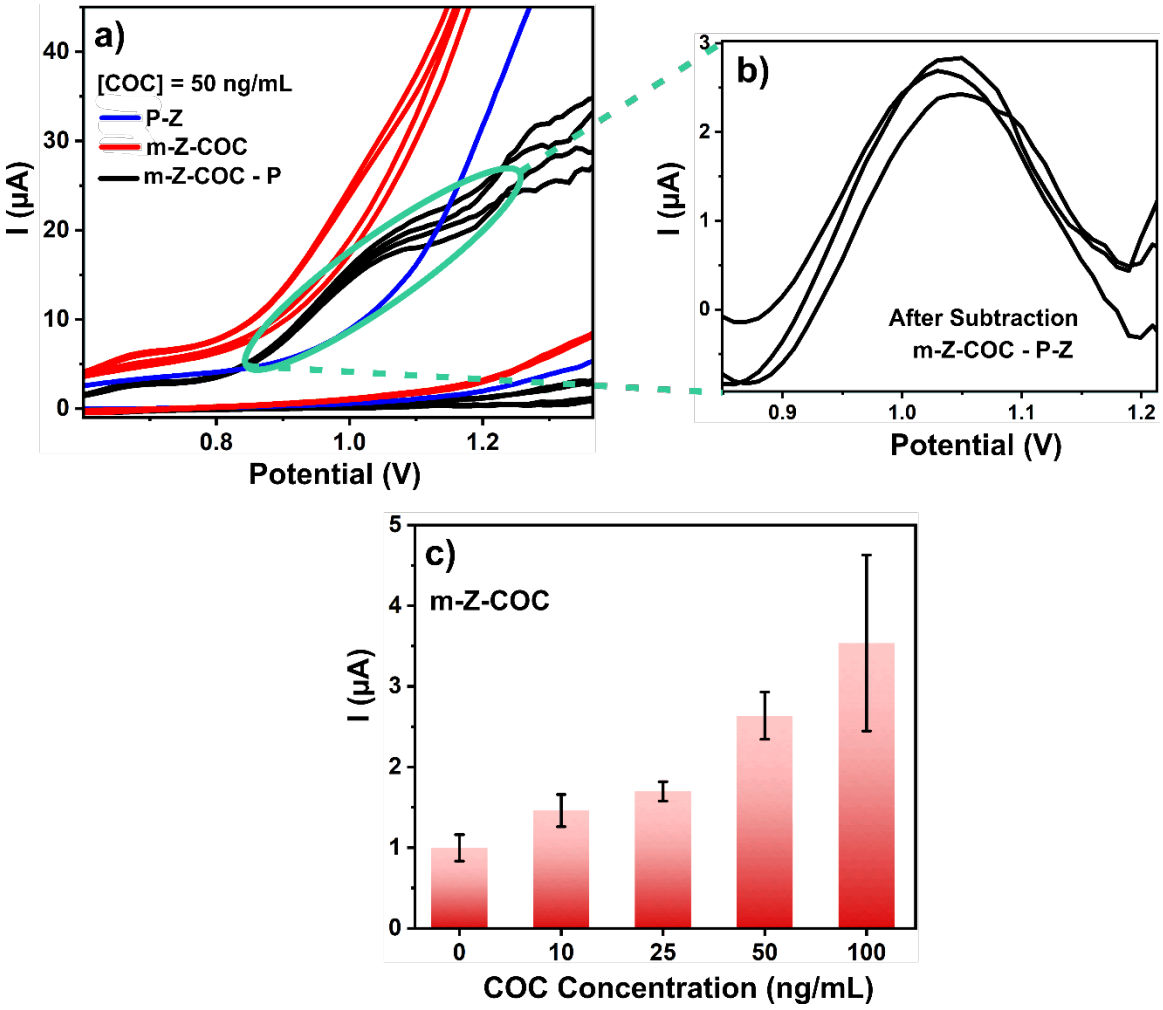


Figure 5-2: (a) and (b) Comparison between original signal and signal subtracted from a spiked P-Z (c) Standard deviation analysis of various concentrations of COC in saliva.

As shown in Figure 5-2 a, there is no signal for the oxidation of COC present. Once the signal from the m-Z-COC is subtracted from the signal of the P-Z, a peak appears precisely where the oxidation peak of COC is expected (Figure 5-2 b). The peak intensity can be visualized after manually evaluating the peak intensity of COC solutions (Figure 5-2 c). However, there is no clear separation between 10 ng/mL and 25 ng/mL and the error bar on 100 ng/mL overlaps with the error bar on 50 ng/mL. The variation that is seen in

Figure 5-3 can be attributed to several factors, including the deposition of the COCi solution and the dispersed concentrations of COC in saliva. Overall, the subtraction method was not efficient nor effective for every saliva sample. While some samples followed a pattern of current intensity correlating with concentration, the data had too much variation. The obstacle of saliva-to-saliva variation was pertinent in this type of analysis, and this is because saliva can vary due to individuals' diets, gender, age etc. Simply using manual calculations was not sufficient for analyzing the existing data set.

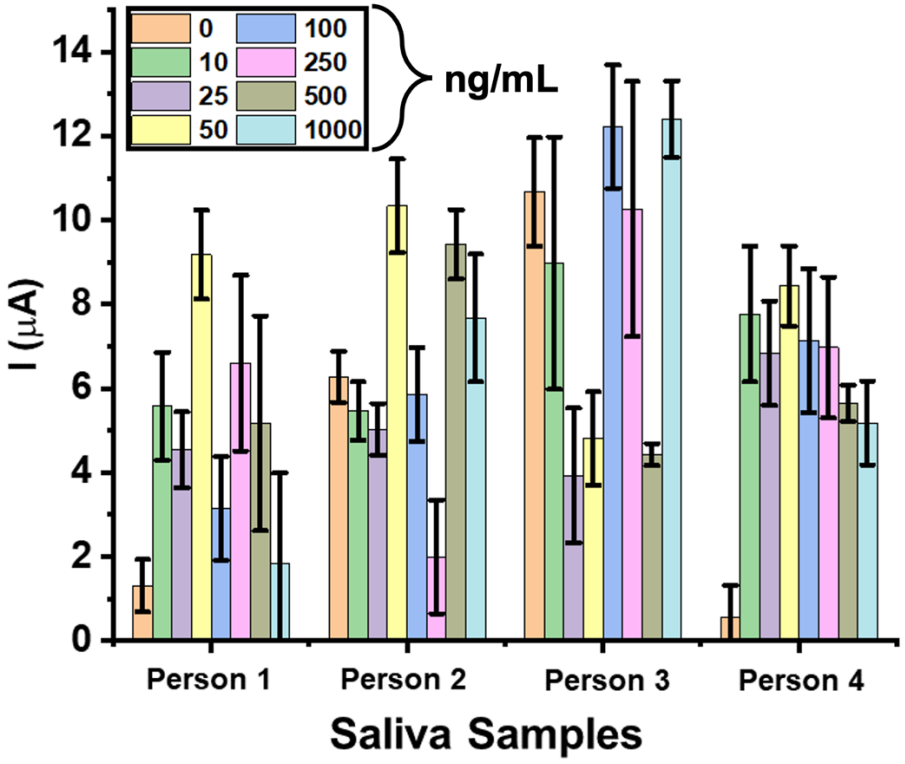


Figure 5-3: Detection of COC in saliva; Data analysis was done by hand. No significant pattern is present, and no conclusions can be drawn from the information.

The introduction of ML algorithms such as Random Forest (RF) and Support Vector Machine (SVM) assisted in eradicating the issues that were commonly found in

electrochemical data, such as anomaly detection, noisy signals and interferents. To further explore the potential of these sensors, ML was introduced as the new method of analysis.

5.4.2 Machine Learning Algorithms

5.4.2.1 Machine Learning in the Detection of COC in Oral Fluid

Manually analyzing the results obtained was not sufficient when working with a complex fluid such as saliva. Similarly, when the data expands after even more testing, it becomes evident that manual calculations are not only inaccurate but also time-consuming. The International Union of Pure and Applied Chemistry (IUPAC) describes an ET as a multisensor system that is composed of several low-selective sensors and uses mathematical procedures for pattern recognition and signal processing [5]. For this application, the multisensor array would be composed of several arrays to detect COC as well as one P-Z to perform the subtraction method. Adding chemometrics to this application will deconvolute the complex and overlapping electrochemical responses obtained and allow for the extraction of meaningful information.

The experimental data were analyzed using RF and Support SVM. RF is an ML method that incorporates a subset of weak learners, such as Decision Trees (DT) to generate a stronger learner. The data is analyzed by training an assortment of independent trees on randomly selected groups of the training set and randomly selected features. Bagging is a technique used in ML to reduce variance found in noisy data. Bagging adds randomness which in turn generates more diversity while decreasing variance. RFs are composed of various DTs and their purpose is to divide data into subgroups that comply with the criteria

set in place for the specific feature. The goal of this is to reduce cross-entropy (S_i) (Equation 5-1) or the sum of region impurity (G_i) (Equation 5-2). In ML, a pure node is a term used to describe when all its data belongs to one single class.

Equation 5-1:

$$S_i = -\sum_{k=1}^{N} P_{i,k} \log\left(P_{i,k}\right)$$

Equation 5-2:

$$G_i = 1 - \sum_{k=1}^{N} P_{i,k}^2$$

Where N is the number of classes and $P_{i,k}$ is the probability of class k among training instances in the i^{th} node.

SVM models are used to separate classes to obtain the most significant margin possible. To do this, hyperplanes are used. Hyperplanes are decision boundaries that aid in the classification of the data points. Support vectors are the nearest points to the hyperplane's margin. The outlier points determine the position and orientation of the hyperplane. Kernel functions facilitate the transformation of data required from its original dimension into a higher-dimension space. The functions base the transformations on the similarity and distances found between two data points in their original dimension. The independent features used as well as the dimension of the data bases have a drastic impact on the performance of SVM methods. In this research Gaussian Radial Basis Function

(RBF) was used as a kernel function.

To analyze the data of various concentrations of COC in saliva, one of the splitting processes used was the Gini impurity approach. This approach is used to decide the most effective split from a root node and other subsequent splits. Although there were eight classes (0, 10, 25, 50, 100, 250, 500, and 1000), only ternary and quaternary classification was performed (Tables 5-1 to 5-4).

Table 5-1: Ternary Classification – Random Forest where C0, C10, and C>=25 represent the concentrations of COC in saliva (0, 10, 25 ng/mL)

P / A	Training			Testing		
	C0	**C10**	**C>=25**	**C0**	**C10**	**C>=25**
C0	33	0	0	5	0	0
C10	0	31	0	1	7	0
C>=25	0	0	32	1	2	8

Table 5-2: Ternary Classification – Support Vector Machine where C0, C10, and C>=25 represent the concentrations of COC in saliva (0, 10, 25 ng/mL)

P / A	Training			Testing		
	C0	**C10**	**C>=25**	**C0**	**C10**	**C>=25**
C0	33	0	0	7	0	0
C10	0	31	0	0	6	2

C>=25	0	0	32	0	3	6

Table 5-3: Quaternary Classification: Random Forest where C0, C10, C25, and C>=50 represent the concentrations of COC in saliva (0, 10, 25, 50 ng/mL)

P / **A**	**Training**				**Testing**			
	C0	**C10**	**C25**	**C>=50**	**C0**	**C10**	**C>=25**	**C>=50**
C0	31	0	0	0	8	0	0	0
C10	0	34	0	0	0	6	0	2
C25	0	0	30	0	0	0	3	0
C>=50	0	0	0	33	1	0	7	5

Table 5-4: Quaternary Classification: Support Vector Machine where C0, C10, C25, and C>=50 represent the concentrations of COC in saliva (0, 10, 25, 50 ng/mL)

P / **A**	**Training**				**Testing**			
	C0	**C10**	**C25**	**C>=50**	**C0**	**C10**	**C>=25**	**C>=50**
C0	31	0	0	0	8	0	0	0
C10	0	34	0	0	1	4	1	1
C25	0	0	30	0	0	1	5	0
C>=50	0	0	0	33	0	1	4	6

Due to the nature of the data, having eight individual classes would introduce too

much variability. The distribution for the ternary classification were 30 (0 ng/mL), 30 (10 ng/mL), 180 (=>25 ng/mL) and for quaternary 58 (0 ng/mL), 40 (10 ng/mL), 40 (25 ng/mL) and 40 (50 ng/mL). Depending on the classification used (ternary or quaternary), the accuracy of the results changed from 78-85% in the testing set and 99-100% in the training set. The highest accuracy with ternary classification came from distinguishing between 0, 10, and 25 ng/mL with 85% accuracy. Using quaternary classification increased the range of concentrations used and the set with the highest accuracy included 0, 10, 25, and 50 ng/mL, obtaining 80% accuracy.

5.4.2.2 Sensor Selectivity

Substances such as caffeine and levamisole are often found in COC as adulterants since they can stimulate the effects of COC and are cheaper [6]. THC and ethanol were also explored since they are substances frequently consumed by COC users [2]. Initially, each drug was individually scanned on various P-Z at a high concentration (50,000 ng/mL) to identify the direct oxidation signal, which would later be beneficial in analyzing the interference with the identifiable peak of COC at 1-1.1V. Levamisole, one of the most used cutting agents (found in over 45% of COC street samples) [6], had a peak very close to COC at 1.16 V with a very high current intensity of 30.3 µA (Figure 5-4 b). After identifying the oxidation peak for each interferent, a smaller concentration of 50 ng/mL of each substance in PBS was scanned on a P-Z.

As expected, such low concentrations of any substance were not visible on P-Z. Still working with PBS, several m-Z-COC were prepared to examine each substance on a

m-Z-COC. Since the electrodes are modified with COC, it is expected that they will have a greater affinity for the COC samples and treat the samples without COC as zeros. However, given that all the substances used are electroactive in nature, it is not rare that their electroactivity would impact the intensity of the current.

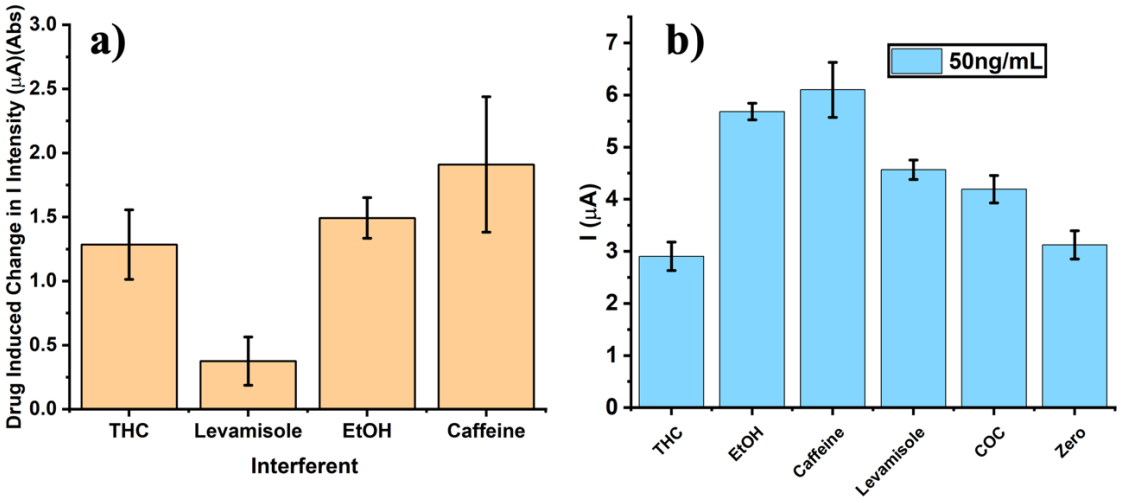


Figure 5-4: (a) Current intensity difference between adulterant and cocaine. (b) Peak intensities are shown in the bar graph where Zero is a blank with no drug present.

To further analyze the affinity of the m-Z-COC to cocaine, the oxidation potential range of cocaine (1-1.1 V) was analyzed as the area of interest. The interferents in saliva were interrogated on m-Z-COC and the difference in current between the interferents and COC can be seen in Figure 5-4. Although there is zero cocaine in the samples interrogated when scanning the different interferents, these samples are inherently electroactive, meaning that their current values were not the same as the current value when interrogating just saliva or just PBS. However, the different oxidation potential of these interferents as well as their different current intensities are a proper indicator that the analyte being detected is not cocaine.

The current intensity of Levamisole appeared to be the most similar to that of cocaine and given that their oxidation potentials are also very similar, ML was also implemented to prove the selectivity of the m-Z-COC. Using binary analysis, it was determined that the model was able to determine between the samples that had cocaine and samples that did not have cocaine (besides the cocaine in the working electrode). For example – one sample of levamisole spiked saliva interrogated on a modified electrode was classified as not having cocaine by the model.

5.4.3 Hook Effect Analysis

Once the current method was established to detect between 0 to 100 ng/mL, it was essential to evaluate if higher concentrations followed the same pattern of current intensity correlating to the analyte concentration. Experiments were performed with concentrations up to 1000 ng/mL (Figure 5-4). The primary goal of this application was to detect from 0 to 50 ng/mL. Therefore, higher concentrations needed to not fall below the intensity of 50 ng/mL.

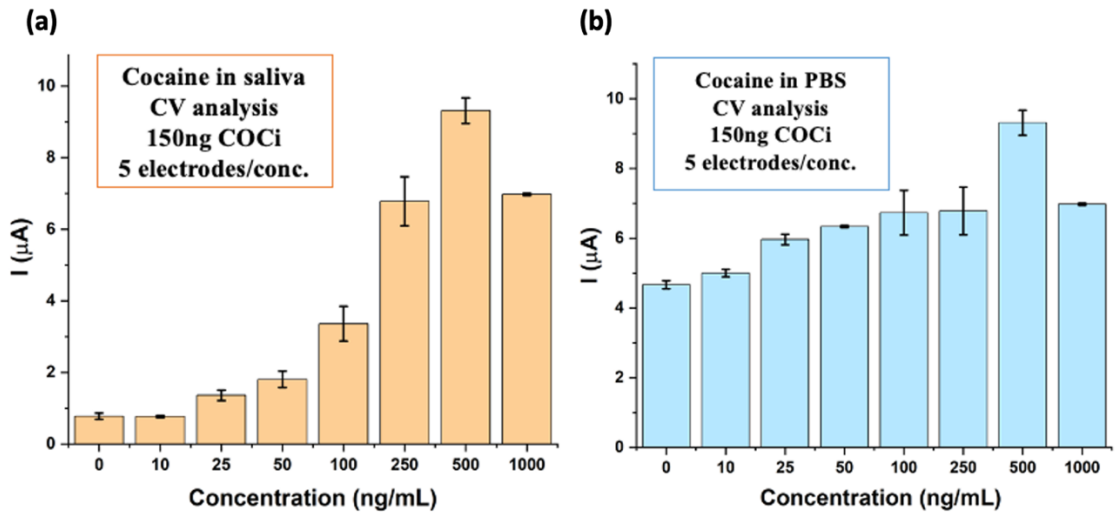


Figure 5-5: Different concentrations of COC in (a) saliva and (b) PBS interrogated under the same parameters and conditions.

Figure 5-5 a shows the hook effect analysis in PBS, and it can be seen that even the current intensity of the highest concentration (1000 ng/mL) does not fall below 50 ng/mL, and the hook effect starts to be noticeable in that range. As explained in chapter 1, the amount of COC present in some individuals following administration is approximately 1200 ng/mL. Similarly, the half-life of COC is very short, meaning that even minutes after consumption, the intact compound begins slowly losing potency and is shortly thereafter excreted from the body. While in PBS, the difference between concentrations is more noticeable, that distinction is not seen (Figure 5-5 a) when evaluating saliva which further proved the need for ML.

5.5 Conclusion

In this study, a novel electrochemical approach for the detection of COC in real saliva

was presented with a low detection range of 0 ng/mL to over 50 ng/mL. The technique uses the physical interactions between the carbon WE and the COC molecule used to modify said WE. When the COC molecules are absorbed on the WE surface, the affinity towards COC samples increases, allowing for the detection of low concentration. While the detection of COC in PBS was straightforward, several issues arose with saliva as it is a recognized complex medium. To eliminate some of the unwanted particles in the oral fluid (such as food), the spiked saliva was filtered. ML methods were also used to analyze the data due to the nature of sensor signals being noisy as well as the saliva interference. Currently, the highest accuracy achieved is 85% with the data presented.

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Chapter 6: Conclusions

6.1 Thesis Summary and Key Findings

6.1.1 Sensor Optimization

One of the focus areas of this research was to find the optimal parameters to modify the chemical sensor to accurately detect COC in PBS or saliva. The modification of the electrodes was a lengthy process with many different parameters to optimize, including:

- The amount of modifying solution (COCi)
- The ratio of liquids in the modifying solution
- The electrochemical technique (and parameters of the technique)
- The stability of the electrodes post-modification (COC oxidation)
- The curing/drying of the m-Z-COC
- pH levels of electrolyte solution
- Latency periods for sample testing

6.1.2 Ensuring Cocaine Detection and Electrode Characterization

COC is an electroactive compound, meaning the drug's direct oxidation is possible on a P-Z. However, COC is not the only electroactive compound used in the experiments. MetOH and PBS, which are key components of the technology, are also electroactive. Since they were part of the modifying solution and testing processes (PBS), experiments were undertaken to ensure that the only molecule being detected and analyzed was COC. This

was achieved via the following steps:

- Verification of the attachment of COC to the WE with ELISA
- Pretreatment of electrodes to eliminate impurities and create a standard electrode surface
- Determining the oxidation potential of COC on a P-Z
- Conducting XPS survey of modified and Z-P
- An experiment comparing the use of PBS vs. H_2O for modifying solution
- Electrochemical characterization using REDOX probe

6.1.3 Cocaine Detection in Spiked Samples (PBS)

Spiked samples of PBS were prepared after the modification and characterization of electrodes were optimized. Samples of 0, 10, 25, 50, 100, 250, 500, and 1000 ng/mL of COC in PBS were prepared and tested with the optimal electrodes and manually analyzed. As presented in Chapter 4, the difference in current intensity was the only parameter investigated in the manual analysis. In theory, higher concentrations of the analyte should result in higher current intensities or, at least, follow some sort of pattern that makes it obvious when different concentrations are analyzed. This pattern was evident when using 150 ng of COCi and detecting 0, 25, 50, and 100 ng/mL of COC in PBS. Also, investigating the hook effect showed valuable information about the range of detection demonstrated by the optimized electrode.

6.1.4 In-Lab Filter Development and Saliva Sensing

Sensing in real saliva was challenging since not much research has been published on the obstacles that can be faced when using the biological fluid in electrochemical analysis. The collection of saliva from lab members was simple and included filtering with glass wool and a cotton pad to absorb the saliva. ELISA was used to quantify the amount of COC left after filtering, and the results were used to move the research forward.

6.1.5 Cocaine Detection in Saliva

Given that saliva and PBS are very different, several parameters were altered to allow for further understanding of how the COC could be detected in this new medium. As explained in Chapter 5, there was no immediate signal present at first sight when implementing the same testing method replacing PBS with saliva. By subtracting the signal from a P-Z from the signal produced by the m-Z-COC, the oxidation signal of COC could be quantified. This method was unreliable because when it was used with different samples, each concentration of COC in saliva had a different current intensity, and the results varied even more between different individuals' samples. To overcome this, ML was employed to analyze the curves since manual calculations could no longer accurately assess the samples. Also, the addition of chemometrics was used to overcome obstacles like:

- Noisy signals
- Saliva to saliva variation
- Human errors from manual analysis
- Other electroactive interferents (isolating only the COC signal)

ML was also used during selectivity analysis to ensure that some of the common adulterants found in COC as well as common drugs did not interfere with the selectivity of the sensor towards COC. Overall, the successful detection of COC was reported in both PBS and saliva with the novel method.

6.2 Field Contributions

- Development of biomolecule-free COC detection
- Detailed explanations of the behaviour of saliva in electrochemical applications
- Electrochemical detection without an incubation period
- Rapid detection with 85% accuracy

6.3 Potential Future Work

1. Automated Modification of Electrodes

For the modification of the electrodes, the deposition of the modifying solution (COCi) was performed manually thorough pipetting. When modifying electrodes, the formation of an even film to coat the WE is one of the most important parameters to optimize. Manual liquid handling (pipetting) introduces errors that result in the variability of the end results. Holford et al. investigated the differences between manual deposition and automated modification using the BioDot ADD3200^{TM} [32]. The BioDot dispensing system uses a micro-needle to dispense liquid on a moving stage. The needle can dispense as little as 20 nL and can dispense an equal amount on each electrode that requires modification. In the experiment performed, automatic deposition using the BioDot has a better linear fit for the

response with an R^2 = 0.98, while manual deposition resulted in R^2 = 0.92. The automatically modified electrode also displayed increased sensitivity and reproducibility.

2. Diversity in Data to Enhance the Machine Learning Algorithm

One of the main limitations of this work is that only ten saliva samples were used. The lab samples obtained were from healthy lab members, and because of this, the variability between the samples was not enough. Most of the lab members were female, and as explained in Chapter 3, saliva samples vary drastically between individuals, especially with different genders, diets, and nationalities. Similarly, to improve the ML algorithm, thousands of data curves are required. Still, this novel research is promising and created a solid foundation for gathering more data to validate further.

3. Interference Analysis

Several studies have been done on the purity of street COC. Although it is known that COC comes from coca leaves, a 2010 study in France reported that from 143 COC samples obtained, the median COC content was 22%, leaving 78% for the adulterants used [33]. This thesis reports interferents used such as caffeine and levamisole and testing has been done with other common mind-altering substances; however, the 2010 study reported paracetamol, diltiazem, and lidocaine. A more recent 2016 study states that only 34% of the street samples were adulterant free [34]. Future research could focus on investigating these in detail.

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