Strategies for Assessing the Limit of Detection in Voltammetric Methods: Comparison and Evaluation of Approaches

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Abstract

The realm of analytical chemistry continues to struggle with defining and evaluating the limit of detection in analytical methods in the sense that a multitude of definitions, criteria, caveats, and methods have been proposed, developed, and adopted across disciplines. The last decade has seen a surge in the growth of electrochemical methods and studies in the field of forensic science and forensic chemistry. While many disciplines within forensic science have established method validation guidelines, the historical and current lack of electrochemical methods within forensic laboratories throughout the United States has left a major gap in knowledge, inhibiting the adoption and utilization of electrochemistry, which may serve as a powerful tool in many subdisciplines of forensics. As such, this work begins this discussion by focusing first on the limit of detection (LOD), with application toward both qualitative and quantitative methods. Both inorganic (ferrocyanide and lead) and organic (diphenylamine, naltrexone, and acetaminophen) target analytes were analyzed via two common voltammetry methods: cyclic voltammetry and square-wave voltammetry. The LOD for each analyte was estimated and/or calculated following a variety of literature-described methods and compared. The accuracy and reliability of these LOD characteristics based on the experimental data is described herein along with suggestions and recommendations. This manuscript is intended to compare the resulting LOD values from various methods and provide a starting point for the incorporation of electrochemistry into the forensic science laboratory, beginning a focused discussion on the development of validation guidelines and parameters needed for the adoption of this technology in forensic laboratories in order to meet the standards required by the criminal justice system.

1. Introduction

Defining the limits of an analytical method is a critical factor for the assessment of the fit-for-purpose of the method and the capabilities of the technique. It would then stand to reason that strict guidelines must be followed when assessing the limit of detection (LOD) to ensure accurate reporting, especially since the LOD is a commonly compared metric between literature works. Despite this, overestimation of the LOD is still commonly seen in reported literature, including works reporting questionable LOD values that are far lower than those of the lowest calibrator. A short review of literature demonstrates that there are a wide variety of definitions and methods for assessing the LOD,[1–8] a fact acknowledged by the Eurachem Guide for Fitness for Purpose of Analytical Methods,[1] resulting in the reporting of differing LOD values. Additionally, this has been a challenge for researchers for decades with improvements and suggestions made for different fields of analytical chemistry.[9–11]

These LOD definitions may also be vague or use terms that are up to the interpretation of the analyst. For example, several common definitions of the LOD follow a format such as "the lowest quantity or concentration that can be measured with a reasonable statistical certainty"[2] or "can be detected with a specified degree of certainty"[3] or "level of confidence."[1] An even more vague definition can be defined as "the level at which the detection of the analyte becomes problematic."[1] While these definitions attempt to encompass the broad range of analytical disciplines, they leave much interpretation up to the end user, which could be a positive outcome but can also result in the reporting of detection limits that do not accurately reflect the capabilities of the method or technique. Some attempts have been made to provide more clarity in LOD definitions through the incorporation of a comparison point such as the following definitions: "an estimate of the lowest concentration of an analyte in a sample that can be reliably differentiated from blank matrix and identified by the analytical method" [4], "lowest concentration that can be distinguished from the background noise with a certain degree of confidence",[5] "lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value",[6] and "the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero."[7]

These definitions provide a comparison point by which to assess the signal from a method, which is a critical component in assessing the LOD, especially in methods with considerable background noise or matrix interference. However, another factor that should be considered is that, by definition, the LOD is assessed at sometimes very low concentrations. Measurements at low concentrations are often subject to higher random errors and deviations from normality assumptions, meaning that the calculation or estimation of the LOD value may also experience large errors. Small alterations in the overall system and performing more experiments or repeated measurements will change the reported LOD, hence LOD can be considered a point estimate.[5,7,8] With these aspects in mind, it stands to reason that a more reliable estimation of the LOD may be obtained under "intermediate precision conditions" such as assessment over multiple runs, days, and samples.[1] This highlights the importance of determining a realistic LOD value, meaning an LOD determined from the entirety of the intended process (sample treatment and preparation, presence of matrix, etc.).[4,11]

While the field of electrochemistry has been prominent since its beginnings in the early 1800s[12–14] and has found footholds in many different scientific disciplines, the use of electrochemical sensors for forensic applications is relatively new, with large growth within the last 10 years to 15 years. The term forensic electrochemistry appears in the literature around 2012/2013; however, electrochemical applications were developed for analytes that could be of forensic use before this time.[15] Although electrochemistry is a mature analytical technique, it is still susceptible to the question of how to best determine the sensitivity of a method. With movement into the science disciplines, it is important that novel methods are presented accurately and with means of comparison to other methods. Voltammetric methods have not yet been incorporated into the categories of analytical techniques by SWGDRUG[16] and they are not yet commonplace in forensic laboratories to the author's knowledge,[17] with the exception of the use of electrochemistry in breath-alcohol testing devices.[18] Despite this, many research groups have touted the advantages of electrochemistry for forensic applications and demonstrated numerous research results including a project in Europe for electrochemical drug detection at borders.[15,17,19,20] Consensus on validation protocols and how to evaluate performance measures is of importance if the final goal is for incorporation into forensic workflows. As such, this work takes a first step toward exploring this

performance measure, the limit of detection, and looks for trends that may suggest a common approach to assessing the LOD in voltametric methods and the potential advantages and disadvantages to the methods used.

2. Commonly Employed Methods

It is no surprise that from the many definitions for the limit of detection come several methods for its calculation and assessment. The best method for estimating the LOD will depend on the application under study. Herein, the focus will be on electrochemical methods involving cyclic voltammetry and square-wave voltammetry. Various LOD calculation methods will be assessed for the electrochemical analysis of inorganic and organic analytes in an effort to determine practical, reliable, and realistic LOD estimations in this field. Although these methods do not encompass all proposed methods in the literature, some of the more commonly employed methods are considered.

2.1. Visual Evaluation

While a purely subjective visual interpretation may lack the objective nature required in analytical chemistry disciplines, this method of interpretation is commonly employed. However, the European Medicines Agency suggests that, in this case, the appropriate data should be presented to justify the visual limit of detection [6]. One example of this approach is a colorimetric test where the generation of color indicates a positive result. Further, a visual approach may be justifiable in electrochemistry. In comparison to some instrumental methods, some electrochemical methods may present baselines with very low noise. In this case, the measurement of any signal (current) may indicate the presence of the analyte and the LOD could be measured visually, where the lowest concentration providing an observable oxidation or reduction peak is reported as the LOD. However, this approach may most often take of the form of serial dilutions compared to a noise value such as section 2.2.

2.2. Experimental Testing/Serial Dilution

As will be discussed later, some references stress the importance of an experimental determination of the LOD value. Often, this procedure is linked to Section 2.1. (due to testing decreasing concentrations, now with the requirement for a quantifiable measurement of the noise) or 2.3. (with the analysis of blank samples and comparison to three times the noise). One of the simplest methods is through the generation of serial dilutions or spiking blank samples at decreasing concentrations. Through this approach, the LOD is taken as the point at which the analyte signal is >3 or >3.3 times the noise. Additional suggestions are offered, such as including three or more fortified blank samples at the suspected LOD and analyzing them in duplicate and repeating over three different runs and assessed as the SNR by **Equation 1**.[4,21]

$$SNR = \frac{analyte\ response}{amplitude\ of\ the\ noise\ (highest-lowest\ point\ in\ baseline\ near\ analyte\ response)} \quad (1)$$

2.3. Measurement of Blanks

Arguably, the assessment of the LOD based on the background signal from the measurement of blanks is one of the most commonly utilized methods and takes several different approaches. Of these approaches, most are familiar with the utilization of the idea of three times the signal-to-noise (or the signal-to-noise ratio, SNR), assuming that the analyte signal intensity is three times larger than the blank signal, which should represent a positive identification. Direct comparison to the noise is used often, where an analyte signal three times the signal of the background noise is used as the LOD (**Equation 2**):

$$LOD = 3 * noise \quad (2)$$

where the $noise$ represents the measured background response and the multiplied constant is represented by 3 or 3.3. The LOD is satisfied when the analyte response exceeds the background response by at least three times (3:1 SNR).[4–6,8] ASB (American Standards Board) Standard 036 for forensic toxicology has provided a statistical method using this approach, requiring three blank matrix samples analyzed in duplicate over three runs, allowing the LOD to be assessed relative to the background as in **Equation 3**:

$$LOD = \bar{X}_B + 3.3 * \sigma_B \quad (3)$$

where $\bar{X}_B$ represents the mean signal of the blank and σ_B is the standard deviation of the blank signal.[4] This type of approach has been suggested in other literature as well.[22] However, it should be noted that in these cases, the LOD is given in the signal domain and not in the concentration domain. To overcome this, several methods of determining the LOD rely on the generation of calibration curve data. It should also be noted that in Section 2.1., the absence of a blank signal or low noise was beneficial for a visual determination of the LOD. In contrast, the lack of a signal from a blank sample, in the case of Section 2.2. and 2.3., is problematic when assessing the LOD using these calculation-based approaches.

2.4. Linear Calibration Curve

A variation on the use of blank samples in combination with the slope of a calibration line is expressed in **Equations 4 and 5**:

$$LOD = \frac{3\sigma_B}{m} \quad (4)$$

$$LOD = \frac{3.3\sigma_B}{m} \quad (5)$$

where σ_B is the standard deviation of the blank signal and m is the slope of the regression line. However, this method clearly requires a calibration curve to be generated, with the requirements for linearity, similar uncertainty across the levels and normally distributed, and multiple measurements.[22] Although the differences between these two equations may seem minimal, they are important. **Equation 4** ensures that the false positive rate is about 0.14 % but allows a false negative rate of 50 %.[2,8] In comparison, **Equation 5** ensures low false positive and false negative rates at 5 % each.[2,8] These equations can also be presented in several other ways by using the standard deviations of the regression line or the standard deviation of the y-intercept.[6] In addition,

ASB Standard 036 recommends that a minimum of three independent calibration curves be included for models that are shown to be linear.[4] Herein, two other variations were tested, replacing σ_B with σ_{L1} (standard deviation of the lowest calibrator) and σ_y (standard deviation of the y-intercept).

2.5. Incorporation of Confidence Limits

It should be clear by now that many factors could influence the determination of the LOD including the method used and the number of samples tested. Therefore, other versions of these calculations have been proposed that incorporate confidence limits and multiplication factors dependent on sample size.[3] The calculation then becomes more involved, as seen in **Equation 6**:

$$LOD = \left(\frac{t(n-2,1-\alpha)s_{yx}}{q_1}\right) * \sqrt{\frac{1+\frac{1}{n}+\bar{x}^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}} \quad (6)$$

where n is the number of levels in the calibration curve, $t(n-2, 1-\alpha)$ represents the t-value for the degrees of freedom $(n-2)$ at the significance level $(1-\alpha)$, q_1 is the slope, $\bar{x}$ is the average concentration of all points in the calibration curve, x_i is the i-th calibration point, and s_{yx} is the residual standard deviation representing the regression error given by **Equation 7**:

$$s_{yx} = \sqrt{\frac{1}{n-2}\sum_{i=1}^{n}(y_i - q_0 - q_1 x_i)^2} \quad (7)$$

where q_0 is the y-intercept.[3] In addition to this example, Benedito da Silva and Machado[23] described the flaws and benefits of the linear calibration approach based on blanks with the use of statistical methods and the residual standard deviation for electroanalysis of paraquat.

In addition to the above method, an attempt at confronting this issue for trace explosives and drug detection resulted in ASTM method E2677-20: Standard Test Method for Estimating Limits of Detection in Trace Detectors for Explosives and Drugs of Interest.[24] This method utilizes an online calculator to determine the LOD(90), LOD(95), etc., for the percent confidence (90 % or 95 %) and the alpha and beta parameters (0.1 or 0.05). Critical to this method is the assessment of at least three different calibration levels, including a zero calibrator, with ten replicates at each level. While this provides a rigorous statistical test of the LOD, it presents some barriers to use. Although only three levels are needed, this is often unsatisfactory, and many areas of analytical chemistry require at least five, non-zero calibrators. This makes the number of tests very large, considering the ten-replicate requirement. Some electrochemical sensors, such as enzymatic biosensors or complex electrode modifications, may be expensive, time-consuming, or intensive to fabricate or modify. This requirement makes testing different methods unfavorable and costly, as such, the ASTM method result will be compared to other methods to understand the effect of the ten replicates in relation to other approaches. Furthermore, more calibration levels in the region of the LOD may be required or suggested by the calculator for improved accuracy and reliability of the calculated LOD. Lastly, a signal at the blank level is required; however, in some electrochemical methods, the blank signal may be zero, presenting a problem for the calculator.

These methods represent common approaches to determining the LOD, not only in electrochemical applications, but throughout forensic chemistry disciplines. These methods were chosen for comparison in this study through the analysis of target compounds that represent both the drug and gunshot residue/explosives areas, as well as common electrochemical scenarios.

3. Experimental

3.1. Reagents and Standards

Lead standard (Inorganic Ventures, Christiansburg, VA) for Inductively Coupled Plasma methods and diphenylamine (DPA) standard from Sigma Aldrich (St. Louis, MO) were used to represent an electrochemical analysis of gunshot residue/explosives. Naltrexone and acetaminophen standards were used to represent an electrochemical analysis of drugs (Cayman Chemical, Ann Arbor, MI). Potassium hexacyanoferrate ($K_4[Fe(CN)_6] \cdot 3H_2O$) (Sigma Aldrich, St. Louis, MO) was used to represent a well-characterized redox couple. Acetate buffer pH 4 (0.1 mol/L) was prepared from glacial acetic acid and sodium acetate anhydrous (Sigma Aldrich, St. Louis, MO) using distilled water. Phosphate buffer pH 6.8 (0.1 mol/L) was prepared from monobasic sodium phosphate and dibasic sodium phosphate (Sigma Aldrich, St. Louis, MO). Potassium chloride (Thermo Scientific, Waltham, MA) was used as a supporting electrolyte.

3.2. Instrumentation and Electrodes

Electrochemical measurements were conducted using the PalmSens4 portable potentiostat (Randhoeve, Netherlands) with PSTrace software (v.5.9.4515). Screen-printed carbon electrodes (SPCEs, DRP-110) served as the analysis platform and were purchased from Metrohm DropSens, USA (Tampa, FL). The SPCEs contained a carbon working electrode with a geometric area of 0.126 cm^2, a carbon counter electrode, and a silver pseudo-reference electrode.

3.3. Sample Preparation and Voltammetric Methods

$K_4[Fe(CN)_6] \cdot 3H_2O$ samples were prepared in phosphate buffer and serially diluted to obtain concentrations of 50 mmol/L, 25 mmol/L, 10 mmol/L, 5 mmol/L, 1 mmol/L, 0.5 mmol/L, and 0.01 mmol/L. Cyclic voltammetry was used for the analysis of $K_4[Fe(CN)_6] \cdot 3H_2O$ to probe the redox couple between $[Fe(CN)_6]^{4-}$ and $Fe[(CN)_6]^{3-}$. The starting potential was -300 mV and was swept first in the anodic direction to +700 mV and then back in the cathodic direction to -300 mV. The potential step was 2 mV with a scan rate of 100 mV/s. Additionally, concentrations of 0.001 mmol/L and 0.0005 mmol/L were prepared and tested.

Lead and DPA were prepared together as previously reported.[25,26] Briefly, an aliquot of the DPA standard was evaporated under air and then reconstituted using acetate buffer and a diluted aliquot of the lead standard to generate a sample containing 4 µg/mL lead and 16 µg/mL DPA. This solution was serially diluted to yield samples containing 2 µg/mL Pb + 8 µg/mL DPA, 1 µg/mL Pb + 4 µg/mL DPA, 0.5 µg/mL Pb + 2 µg/mL DPA, 0.25 µg/mL Pb + 1 µg/mL DPA, and 0.125 µg/mL Pb + 0.5 µg/mL DPA. Additional samples were prepared to contain 0.05 µg/mL Pb + 0.5 µg/mL DPA, 2 µg/mL Pb and DPA, 0.2 µg/mL Pb and DPA, and 0.02 µg/mL Pb and DPA. Square-wave anodic stripping voltammetry (SWASV) was utilized to analyze GSR-related compounds,

Pb and DPA. The method parameters were employed based on Dalzell et al.[26] and were as follows: deposition potential -950 mV, deposition time 120 s, starting potential -1000 mV, ending potential +1200 mV, potential step 5 mV, amplitude 25 mV, frequency 11 Hz. The deposition stage serves as a pre-concentration stage prior to the square-wave sweep.

Naltrexone samples were prepared in the same manner as acetaminophen but at concentrations of 100 µg/mL, 80 µg/mL, 60 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, and 1 µg/mL with two additional samples at 6 µg/mL and 3 µg/mL Square-wave voltammetry was also employed for the detection of naltrexone based on previous experiments. The starting and ending potentials were -1000 mV and +1200 mV, respectively, with a potential step of 15 mV, amplitude of 100 mV, and a frequency of 100 Hz.

Acetaminophen samples were prepared by first creating an initial stock concentration by aliquoting the organic standard and evaporating. This sample was then reconstituted in water with 0.1 mol/L potassium chloride. Concentrations of 10 µg/mL, 6 µg/mL, 3 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.2 µg/mL, and 0.1 µg/mL were prepared and tested. Square-wave voltammetry, utilizing an anodic sweep and a cathodic sweep, from previous experiments was used. The starting potential was -600 mV and was swept to +1000 mV with a potential step of 4 mV, amplitude of 250 mV, and a frequency of 75 Hz. This procedure was then reversed to start at +1000 mV and end at -600 mV to obtain the reduction peak.

3.4. Data Analysis

The PSTrace5 software (v.5.9.4515 build 26368 f) was used for initial data analysis using a combination of automatic peak detection by the software and manual peak selection (selecting the baseline across the peak and the apex of the peak) to obtain peak heights (peak current) and/or the integrated areas of the oxidation and reduction peaks obtained from the voltammetric methods. For cyclic voltammetry, peak height from the extrapolated baseline was used for statistical measures, whereas the integrated peak area was used for square-wave methods. Following the integration and selection of oxidation and reduction peaks in the software, all data were exported to Microsoft Excel (version 2308 Build 16.0.16731.20542) for calculation of limits of detection. The methods outlined in Section 2 were used for the assessment of the limit of detection, where the following methods were denoted by letter: LOD by noise (A) (signal domain), LOD by blank (B) (resulting in information in the signal domain, but for comparison purposes, the signal was passed through the regression equation to obtain the LOD in the concentration domain), LOD by standard deviation of the blank (C), LOD by standard deviation of the lowest calibrator (D), LOD by standard deviation of the y-intercept (E), LOD by the 99 % confidence limit (F), and LOD by ASTM method for the LOD(95) (G) (performed for lead and diphenylamine). Method A provides an estimation of the LOD in the signal domain. For these experiments, the value in the signal domain for method B was similar to the noise measurement via method A, supporting the conversion of the LOD value from method B into the concentration domain for comparison purposes. Experimental testing of the LOD via serial dilution was used as a comparison for the calculated LOD values and was reported as a range based on comparison to the noise value, providing the point between the disappearance of the signal and the next highest tested

concentration. If the lowest tested concentration was above three times the noise, then the LOD was reported as less than that number.

4. Results and Discussion

4.1. Cyclic Voltammetry of Ferrocyanide: A Common Redox Probe Species

One of the first electrochemical experiments performed in many applications is cyclic voltammetry, providing information regarding the electroactivity and properties of the target analyte. If following traditional cyclic voltammetry parameters, both an oxidation and reduction peak will be present for reversible systems, resulting in the first question regarding the determination of the limit of detection. Should the LOD be assessed using the oxidation peak, reduction peak, or a combination of both? To explore this application, a classic redox couple was chosen for analysis, $K_4[Fe(CN)_6] \cdot 3H_2O$, for the reversible pair $[Fe(CN)_6]^{4-} \rightleftharpoons Fe[(CN)_6]^{3-}$. **Figure 1** demonstrates the cyclic voltammograms generated from the calibration samples tested over the range of 0 mmol/L to 50 mmol/L. Peak heights (currents) were reported as commonly measured in cyclic voltammetry experiments.

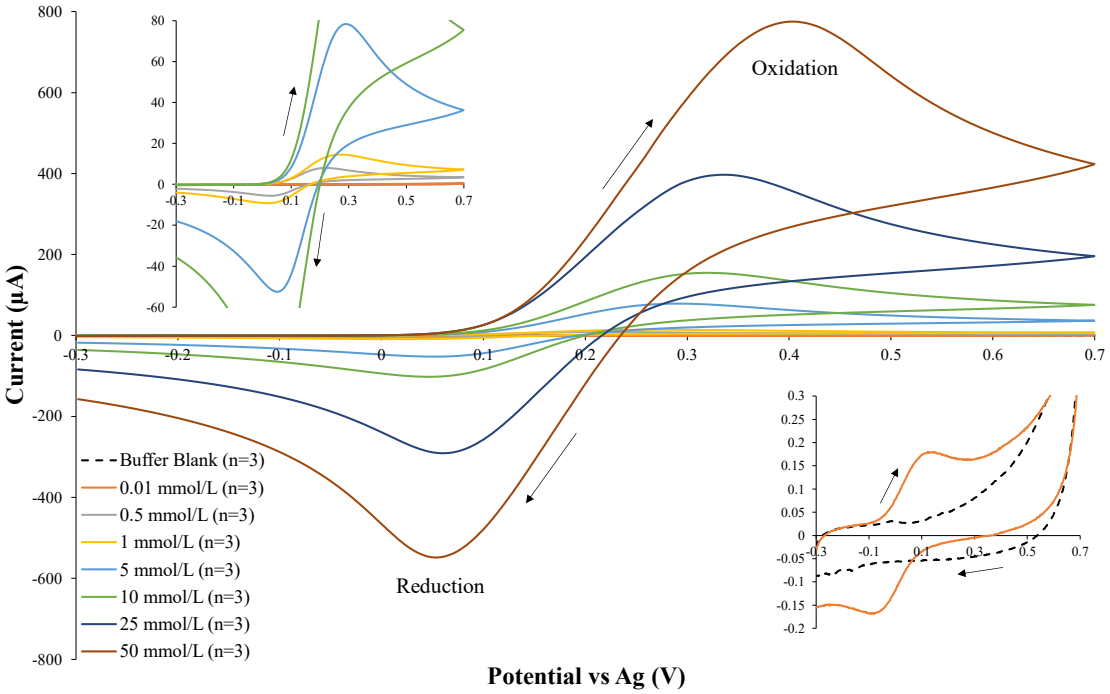

Figure 1. *Average cyclic voltammograms for the redox couple $[Fe(CN)_6]^{4-} \rightleftharpoons Fe[(CN)_6]^{3-}$ in 0.1 mol/L phosphate buffer pH 6.8 over the range of 0 mmol/L to 50 mmol/L performed in triplicate at a scan rate of 100 mV/s as a 50-microliter drop on the electrode surface. Inlays demonstrate zoomed in areas of the cyclic voltammograms for ≤ 5 mmol/L (top left) and ≤ 0.01 mmol/L (bottom right).*

Initially, it can be noted that the potential difference between the oxidation peak and the reduction peak is larger than expected, suggesting uncompensated resistance within the screen-printed carbon electrodes. (**Supplemental Figure S1)**. However, the peak heights were similar, although the reduction peak was slightly smaller across all calibrators. The average potential of the oxidation

peak and reduction peak was found to be +281 mV ± 88 mV and +32 mV ± 42 mV, respectively. Excellent linearity was observed for both the oxidation peak and reduction peak. **Figure 2** provides the calibration curves obtained for the forward and reverse scans demonstrating R^2 values greater than 0.99.

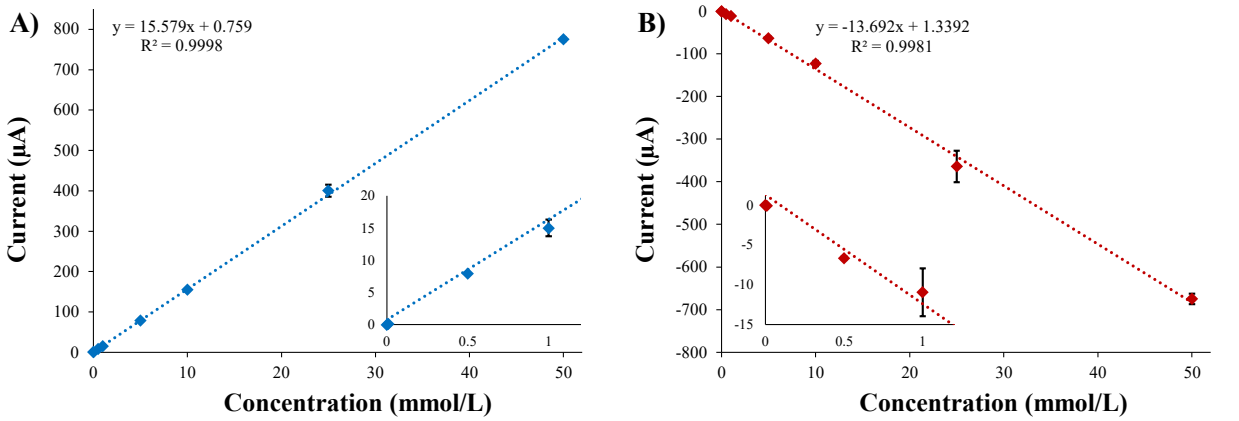

Figure 2. *Calibration curves for triplicate analysis of the redox couple* $[Fe(CN)_6]^{4-} \rightleftharpoons Fe[(CN)_6]^{3-}$ *for A) the oxidation peak and B) the reduction peak based on peak height. Inlays demonstrate a zoomed-in region at the lower end of each calibration curve. Error bars represent the standard deviation.*

Although excellent linearity was achieved over the calibration range, when observing the residuals across the calibrators, heteroscedasticity was evident considering the range of concentrations in the curve (**Supplemental Figure S2**). As a result, the linearity of the calibration curve introduced error at low-concentration calibrators, resulting in an erroneous calculation of the LOD when determining the limit based on the signal of the noise in the blanks (a negative value was obtained). Heteroscedasticity can be corrected mathematically by employing a weighting factor to the curve, where two common weighted linear regressions are 1/x and $1/x^2$.[27–29]

The use of weighted regression statistics is common practice in some disciplines of analytical chemistry such as methods employing liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry since measurements can often span several orders of magnitude.[28,30] However, the use of weighting factors for electrochemical measurements seems to be rarer, as mentions of using weighted linear curves are not often found in the literature. Despite this, electrochemical techniques typically display high sensitivity, and instances with wide dynamic ranges may result in heteroscedasticity.[29] In these cases, it may be favorable to apply weighted regression to achieve more accurate concentration estimations across the working range.[28] . Exceptions may be observed when the calibration range is small. Utilizing this approach and the R package CCWeights, the appropriate weighting factor was selected based on minimizing the sum of the absolute relative error and then applied to the curve. **Supplemental Figure S3** demonstrates the comparison between the unweighted regression and 1/x weighted regression for the oxidation peak. Although not much difference is observed for the calculated concentrations over the entire range, improvements are noticeable in the lowest concentrations. Using the weighted curve, the LOD calculation using the blanks now resulted in a reasonable estimation of the LOD (**Table 1**). Additionally, the LOD estimate for method E was corrected due to an improved y-intercept value. Since weighted curves are not common in the literature, the effect of

applying weighted curves was not assessed with the other analytes in order to mimic typical experimental results.

Following the outlined methods of LOD calculation, **Table 1** provides the various LOD values obtained from the experiments. There are clear differences between the LOD values obtained using the various methods and, in some cases, significantly different values. A difference of two orders of magnitude were seen between methods E and F compared with the other methods. The visual LOD via serial dilution was determined to be between 0.001 mmol/L and 0.01 mmol/L, with a very small signal visible in the 0.001 mmol/L sample, suggesting accurate LOD calculation for methods A, B, C, D, and E (weighted) with method D providing a more realistic estimate within the experimentally observed LOD range. Method F has clearly underestimated the detection capability of the method since the calculated LOD value is larger than the lowest calibrator in the curve that provided adequate signal. A visual comparison of these data can be observed in **Figure 3**. Similar trends were observed for the reduction wave, where the weighted curve, in this case $1/x^2$, resulted in more realistic estimation of the LOD using methods B and E (**Supplemental Figures S4 and S5 and Table S1**). In the case of ferricyanide, the use of a set of blank signals allowed for the calculation of an LOD value comparable to experimental results through serial dilution.

Table 1. *Assessment and comparison of the LOD, in mmol/L for comparison to literature, for the redox couple* $[Fe(CN)_6]^{4-} \rightleftharpoons Fe[(CN)_6]^{3-}$ *via cyclic voltammetry, using the various reported methods in the literature for the oxidation peak compared between the weighted (1/x) and unweighted linear curves. Method A is reported in µA since the noise is measured in the signal domain as peak height.*

	Method	Unweighted	Weighted
A	$3 * Noise$	0.0240 µA	
B	$\bar{X}_B + 3.3 * s_B$	-0.0471	0.0012
C	$(3.3 * \sigma_B)/m$	0.0012	0.0012
D	$(3.3 * \sigma_{L1})/m$	0.0050	0.0050
E	$(3.3 * \sigma_y)/m$	0.1288	0.0012
F	99 % C.L.	0.1447	0.1586
Experimental	Serial Dilution	0.001 < LOD < 0.01	

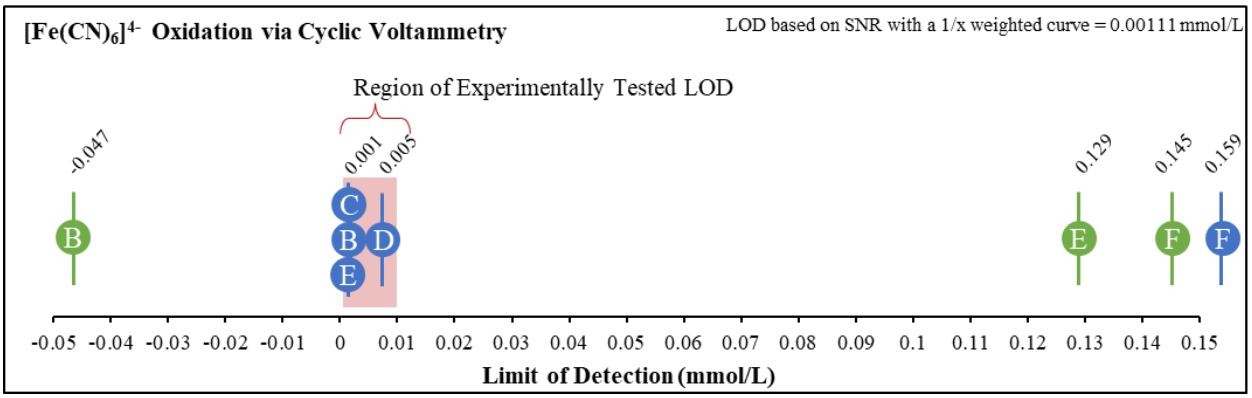

Figure 3. *Graphical comparison of the LOD values for the oxidation peak of the redox couple* $[Fe(CN)_6]^{4-} \rightleftharpoons Fe[(CN)_6]^{3-}$ *in relationship to the experimentally tested LOD value for both the weighted (1/x) (blue) and unweighted curve (green).*

However, there are two other challenges faced when dealing with voltammetric measurements. First, many electrochemical techniques are extremely sensitive for their analytes of interest, meaning that blank samples may not produce any discernible signal. How might this affect the calculation of LOD values in these cases? Second, while cyclic voltammetry may allow for the determination of the noise in the region of interest, other voltammetric techniques are designed to greatly reduce or eliminate noise. For example, the current sampling points during a square-wave voltammetry experiment are set to reduce signal from non-faradaic current, essentially greatly minimizing the noise in the measurement and producing current related only to the faradaic processes that are occurring.

4.2. SWASV of Lead and Diphenylamine: Inorganic and Organic Analytes

Many electroanalytical methods utilize square-wave voltammetry for its exceptional detection capabilities resulting from improved signal-to-noise due to when the current response is sampled. This minimizes current contributions from non-Faradaic processes. To investigate these scenarios, lead and diphenylamine were measured using square-wave anodic stripping voltammetry (**Figure 4**). The analysis of lead and diphenylamine represent analytes of interest to forensic gunshot residue, explosives, and exposure testing and determination. Some challenges in these areas that have been faced in forensic context is the simultaneous detection of heavy metal analytes with organic residues. These forensic applications using electrochemistry have been under study for a number of years as detailed in the reviews by Harshey et al.[31] and O'Mahony and Wang[32]. Several other research groups have investigated these analytes including for diphenylamine,[33] for lead,[34–38] and for both.[25,26,39–41]

Calibration curves were obtained over two different ranges for each analyte based on past experience and method sensitivity and were reported as peak areas. In comparison to previous literature,[25,26,39] the oxidation potentials for each analyte were in agreement and demonstrated small standard deviations, with potential response changing logarithmically as expected (**Supplemental Figure S6)**. The oxidative electrochemical potential was determined to be -777 mV ± 26 mV for lead and +434 mV ± 16 mV for diphenylamine.

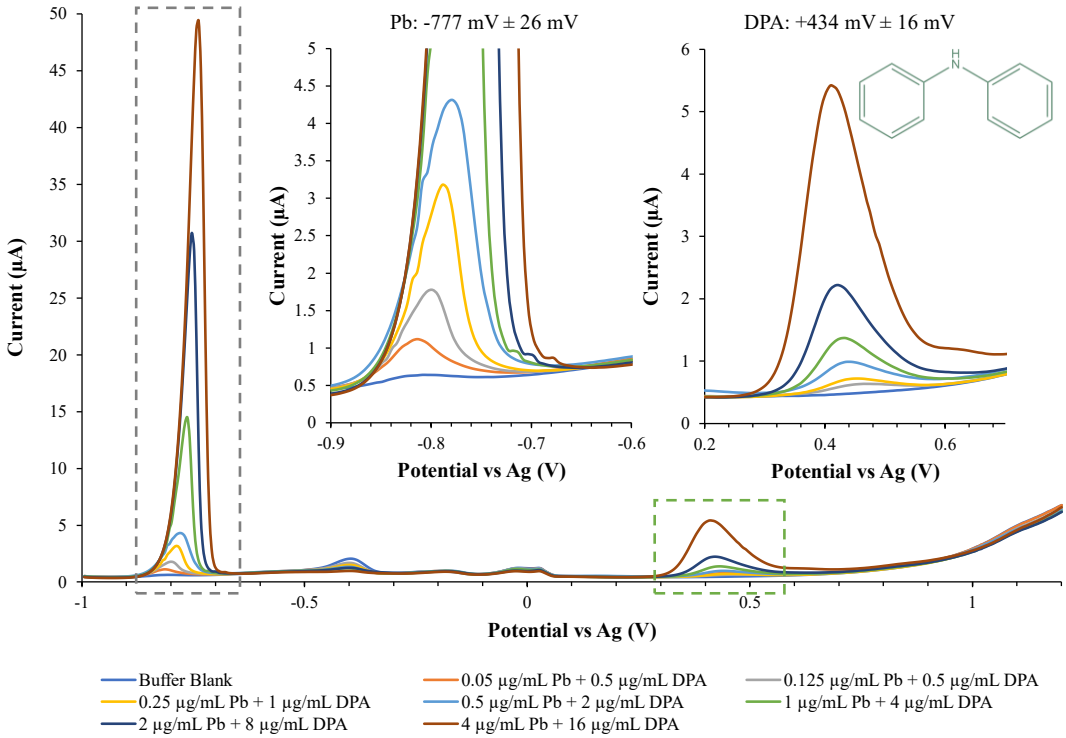

Figure 4. *Square-wave anodic stripping voltammograms of a binary mixture of lead (range = 0 µg/mL to 4 µg/mL) and diphenylamine (range = 0 µg/mL to 16 µg/mL). Average voltammograms for 10 replicates at each level. The inlays demonstrate the zoomed-in regions corresponding to the lead (left) and diphenylamine (right), along with their observed oxidation potential.*

Ten replicates at each of the concentration levels (8 levels for lead and 7 levels for DPA) were assessed. Loss of linearity was observed for DPA at the highest concentration of 16 µg/mL. Therefore, the range 0 µg/mL to 8 µg/mL was used. The sensitivity of the method was higher for lead than for DPA, as evidenced by the voltammograms and slopes of the calibration curves, suggesting almost 25 times greater sensitivity (**Figure 5)**. The limit of detection determination for lead and diphenylamine demonstrates two different aspects. During the analysis of the blanks, a small oxidative signal was observed in the potential area of the lead oxidation peak, allowing for the blank signal methods to be used. In this case, the average blank signal was also used as the noise measurement to determine the signal at three times the signal-to-noise since a discernible value for the noise could not be determined using this SWASV method. The breakdown of the LOD determinations is given in **Table 2** with graphical depictions in **Figure 6**.

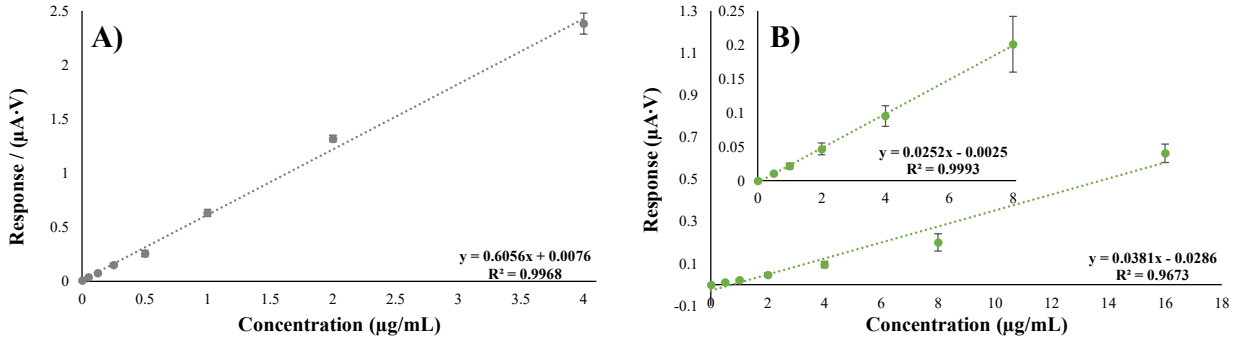

Figure 5. *Calibration curves for the peak area for A) lead and B) diphenylamine using square-wave anodic stripping voltammetry for n=10 replicates. Inlay demonstrates the calibration curve with the highest calibrator dropped due to a departure from linearity. Error bars represent the standard deviation.*

Table 2. *Limit of detection values, in µg/mL, determined for lead and diphenylamine measured by square-wave anodic stripping voltammetry. Method A contains units of µA·V for the signal domain as measured by the area of the peak.*

Method		Lead	Diphenylamine
A	$3 * Noise$	0.0243 µA·V	0.00006 µA·V
B	$\bar{X}_B + 3.3 * s_B$	0.0175	0.2821
C	$(3.3 * \sigma_B)/m$	0.0175	0.0973
D	$(3.3 * \sigma_{L1})/m$	0.0213	0.1999
E	$(3.3 * \sigma_y)/m$	0.0371	0.3702
F	99 % C.L.	0.0280	0.3119
G	ASTM E2677-20	0.0187	0.2078
Experimental	Serial Dilution	0.02 < LOD < 0.20	≈0.20

Although the differences between the calculated LOD values do not appear to be large, when graphically represented, the discrepancy between certain LOD values with the experimental data is apparent. At a concentration of 0.02 µg/mL, the lead signal either could not be differentiated from the blank signal or was very similar to the blank signal (less than three times the blank signal). Therefore, the calculated LOD values below this concentration would be overestimating the ability of the method to detect lead. This also includes the LOD(95) method by ASTM E-2677, which provided one of the lowest LOD values of 0.0187 µg/mL. However, it should be noted that the reported 95 % upper confidence limit on the LOD was approximately 0.04 µg/mL via ASTM E-2677, which could be a more accurate representation of the LOD.

Concerning diphenylamine, the problem regarding the absence of a blank signal is quite obvious. Using SWASV, no signal was recorded for any blank sample at the expected electrochemical potential, preventing the use of these methods otherwise providing an LOD value of 0, which is unrealistic due to the many variables involved in a measurement (although a very small electrochemical peak was observed in half of the blank samples at a potential before the oxidation of DPA). However, a noise measurement was estimated manually over several voltammograms using the peak measurement tools in the software for the SWASV method as $2x10^{-5}$ µA·V. Methods B, D, F, and G provided the most realistic assessments of the LOD. In the case of lead and diphenylamine, more measurements were requested by the ASTM calculator in the vicinity of the lower calibration region.

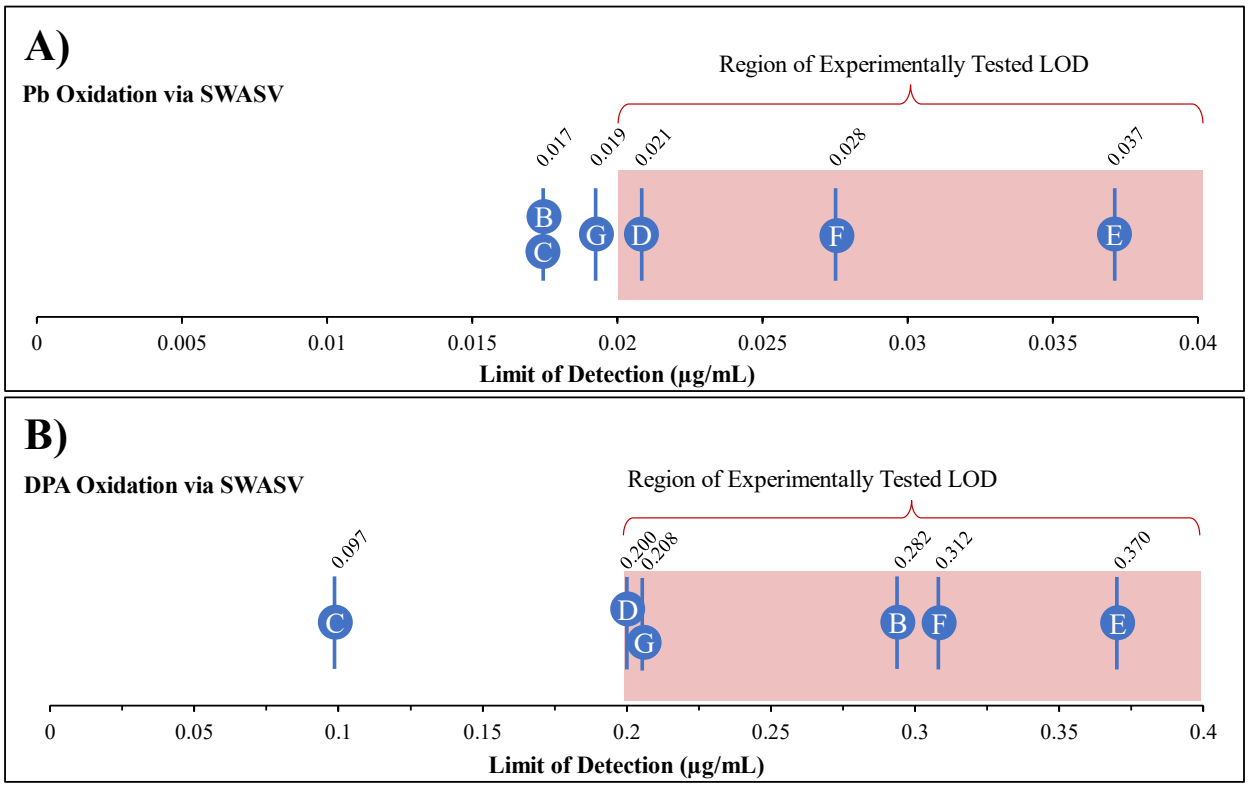

Figure 6. *Graphical depiction and comparison of the LOD values determined for A) lead and B) diphenylamine using the various approaches.*

4.3. SWV of Naltrexone: Multiple Oxidation Peaks

Another question of importance is how to treat an analyte that produces multiple oxidation or reduction peaks. This question comes down to a discussion revolving around the identification criteria for the method. It is possible that the developed sensor may be specific enough that only the most sensitive peak is needed for identification. This may also be the case since more than one oxidation peak could represent two consecutive electrochemical processes. However, others may argue that identification of the analyte requires the presence of all peaks to reliably determine the analyte from other similar materials.[42] One example of this can be seen in the electrochemical analysis of naltrexone via square-wave voltammetry, demonstrating two oxidation peaks, one at 168 mV ± 8.4 mV (Peak I) and the other at 752 mV ± 44 mV (Peak II) (**Figure 7**). Naltrexone is an organic drug molecule, representing the analysis of drug substances in forensic and clinical applications. Naltrexone, an opioid receptor antagonist, represents a medication-assisted treatment therapy for treating opioid abuse and alcohol abuse.[43] Electrochemical detection of naltrexone has been reported in several studies.[44–48]

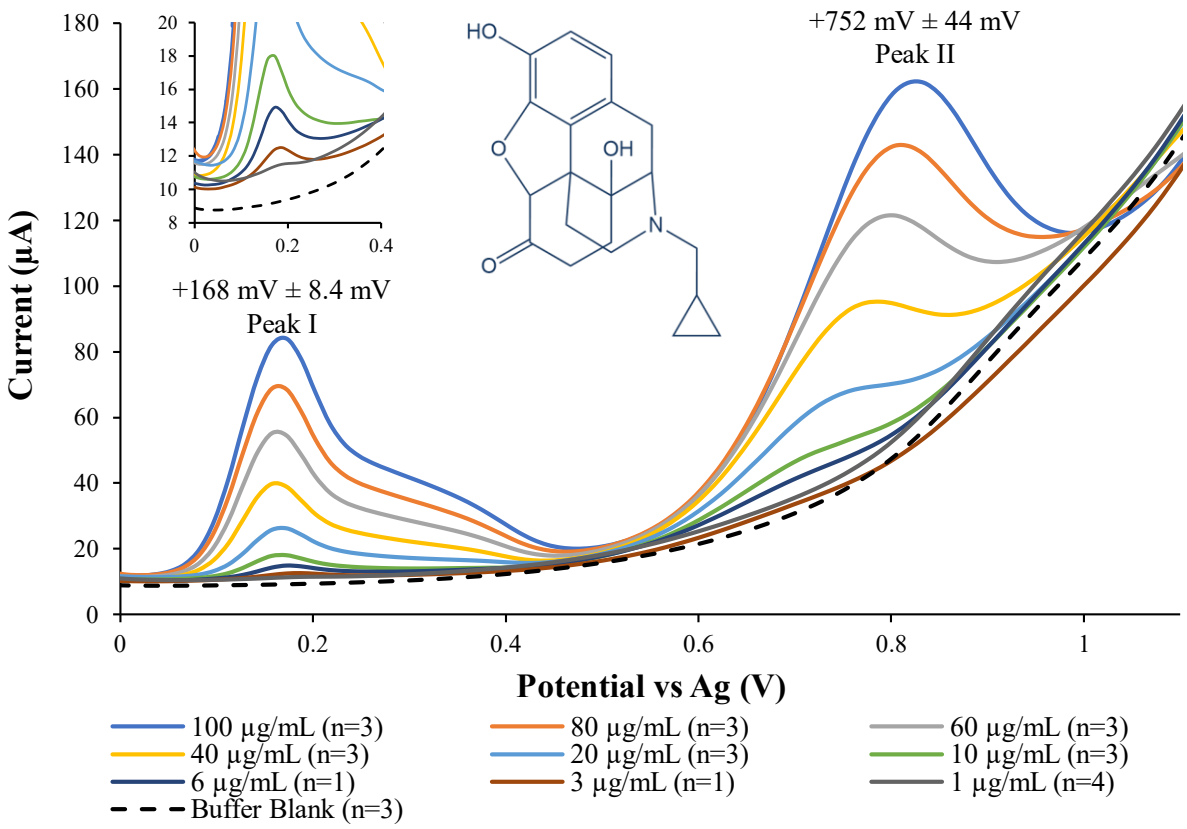

Figure 7. *Average square-wave voltammograms (n=3) constructed from the calibration samples for the analysis of naltrexone, demonstrating two oxidation peaks and regions of interest. Analysis was performed in 100 mmol/L KCl.*

Linearity was observed for oxidation Peak I (R^2 = 0.9982) and for oxidation Peak II (R^2 = 0.9852). The slight loss of linearity for oxidation Peak II may be attributed to the higher oxidative potentials being applied in the region of the peak, leading to the growing background current related to the generation of oxygen from the oxidation of water from the electrolyte. This background current and skew of the baseline lowers the signal-to-noise ratio for oxidation peaks in the area.

Of interest to this study is how the sensitivity of the two oxidation peaks compares to each other (**Table 3**). Oxidation Peak I demonstrated higher sensitivity for the naltrexone compound, where the experimental test showed that the LOD was lower than the 1 µg/mL calibrator, whose response was approximately 0.01 µA·V, two orders of magnitude higher than three times the noise (0.0006 µA·V). With this in mind, the only method producing a reasonable estimate of the LOD was method D (using the standard deviation of the lowest calibrator), whereas the other methods resulted in underestimating the sensitivity of the method and produced LOD values greater than that which was tested experimentally. However, with regard to oxidation Peak II, the sensitivity was much worse and the experimental LOD was determined to be between 3 µg/mL and 6 µg/mL. In this case, only method F (99 % confidence limit) produced an LOD value within this range. Further, the 6 µg/mL calibrator gave a response of approximately 0.04 µA·V, supporting the identification of this peak compared to the noise measured. The graphical depictions of the LOD values can be found in **Supplemental Figures S7 and S8**. As both peaks represent oxidation of the molecule, the question becomes how to report the LOD, which will be influenced by what the

method is intended for. It seems reasonable that the LOD may be reported as approximately 0.5 μg/mL with quantitative ability above 4 μg/mL (although this level would need to be tested). This assumes that in the absence of interfering species, observation of oxidation Peak I would provide identification of naltrexone, but the additional peak would be required for confirmation and quantitation. The other approach would be to err on the conservative side and report the higher LOD of the two peaks.

As mentioned earlier, oxidation Peak I allowed for detection of lower concentrations, while oxidation Peak II suffered due to the rise in background current. This background current may have also played a role in producing calibrators with responses that fell below the linear regression line. As a result, the y-intercept values deviated significantly from zero, further demonstrating the loss of sensitivity (**Supplemental Figure S9**). As such, since no blank signal was measured, a value of zero was entered for method B (average blank signal) for both peaks, which was then calculated through the equation of the line of best fit to provide the LOD result in the concentration domain. Because of the above-mentioned factors, method B does not provide a trustworthy result. Lastly, so far it has been seen that method F provides a more conservative estimate of LOD with the incorporation of the 99 % confidence limit. In this case, method F may be considered the best approach given the deviation from linearity and skewed y-intercept value.

Table 3. *Limit of detection values, in μg/mL, determined for naltrexone measured by square-wave voltammetry for oxidation Peak I and Peak II. Method A contains units of μA·V for the signal domain as measured by the area of the peak.*

Method		Peak I	Peak II
A	$3 * Noise$	0.0006 μA·V	
B	$\bar{X}_B + 3.3 * s_B$	5.9804	12.9161
C	$(3.3 * \sigma_B)/m$	N/A	N/A
D	$(3.3 * \sigma_{L1})/m$	0.4789	0.8350
E	$(3.3 * \sigma_y)/m$	1.0981	2.1112
F	99 % C.L.	1.5859	4.3040
Experimental	Serial Dilution	LOD < 1	3 < LOD < 6

4.4. SWV of Acetaminophen: Reduction and Oxidation

The final analyte of interest was acetaminophen and the question of how to treat the LOD for an analyte that produces both an oxidation peak and a reduction peak (**Figure 8**). Acetaminophen, also known as paracetamol, is a well-known, studied, and commonly encountered pharmaceutical in the clinical and forensic settings. Much work has been performed with this molecule and recent examples demonstrate its electrochemical behavior.[49–52] Aside from its clinical use, acetaminophen is also of interest in forensic applications due to its presence in the drug supply as an adulterant.[53–55] In comparison to ferricyanide, acetaminophen represents a more challenging analyte in terms of its electrochemical behavior. The oxidation and reduction peaks were comparable in area. Oxidation peak areas were slightly larger than their reduction peak counterparts with the exception of the 0.2 μg/mL level, where the reduction peak was easily visible, but the oxidation peak was barely over three times the noise. The oxidation potential for acetaminophen was determined to be +153 mV ± 8 mV and the reduction potential was +198 mV ± 7 mV. This slight positive shift for the reduction wave most likely resulted from the separate application of the forward and reverse

waves as required by the potentiostat software. No signal was seen in the 0.1 µg/mL lowest level calibrator, resulting in a response of 0 µA·V in method B. Excellent linearity and repeatability was observed for the curves measured in triplicate (**Supplemental Figure S10**).

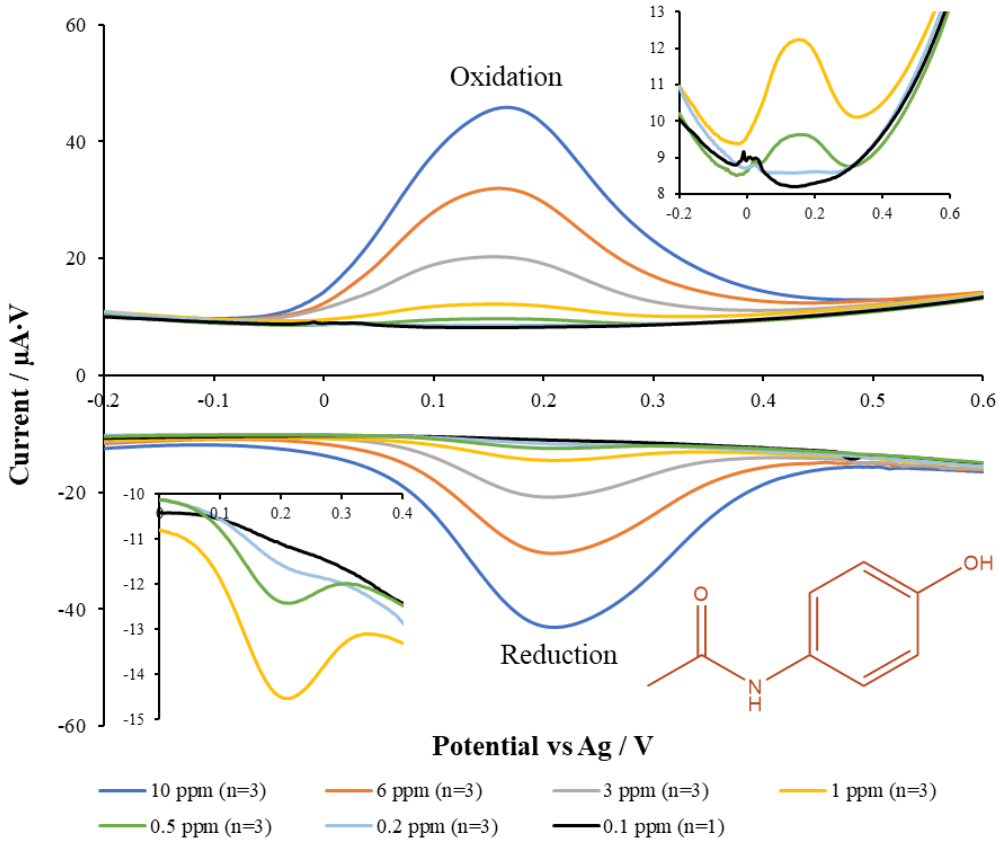

Figure 8. *Average square-wave voltammograms for acetaminophen showing the oxidation wave and reduction wave (n=3). Top inset shows the zoomed in area of the oxidation peak and the bottom inset shows the zoomed in area of the reduction peak.*

More experiments would be needed at intermediate concentrations to provide a better estimate of the LOD values within the experimental range; however, one note that can be made for acetaminophen is in method D that predicted an LOD value outside of the experimental range (**Supplemental Figures S11 and S12**). This was a result of a significantly low standard deviation of the replicants collected at the lowest concentration range, which appeared to be disproportionate to the other calibrators. However, in general, both analytes in this study that resulted in both oxidation and reduction peaks have demonstrated correlation between the LOD estimates for both electrochemical peaks. As such, the LOD value may be chosen to be reported as the average between the oxidation and the reduction peak if requiring both peaks for identification or either peak depending on the method and identification requirements that are set forth. Additionally, a more conservative approach would be to report the higher value (**Table 4**). Interesting, methods E and F provided estimations that aligned better with the experimental value.

Table 4. *Limit of detection values, in μg/mL, determined for acetaminophen measured by square-wave voltammetry for both the oxidation peak and the reduction peak. Method A contains units of μA·V for the signal domain as measured by the area of the peak.*

Method		Oxidation	Reduction
A	$3 * Noise$	0.0007 μA·V	0.003 μA·V
B	$\bar{X}_B + 3.3 * s_B$	0.3409	0.3806
C	$(3.3 * \sigma_B)/m$	N/A	N/A
D	$(3.3 * \sigma_{L1})/m$	0.0315	0.0241
E	$(3.3 * \sigma_y)/m$	0.2612	0.2320
F	99 % C.L.	0.1121	0.1011
Experimental	Serial Dilution	≈ 0.2	0.1 < LOD < 0.2

4.5. Summary

In summary, several different types of analytes and techniques have been presented here and analyzed over varying concentration ranges. In general, it was shown that the square-wave voltametric methods produce voltammograms of low noise, making the determination of the noise in the measurement difficult and resulting in a small noise measurement for method A (noise approach). Method B (average blank signal) is related to method A in the fact that in many electrochemical measurements, the analysis of blank samples may not produce any discernible signal in the potential region of interest. In these cases, any signal measured would be a result of the noise in the system. This makes method B susceptible to being influenced disproportionately by the regression values since the signal of the blank would be either zero or close to zero. However, method A is useful in the fact that it provides an estimation in the signal domain. While this does not directly aid in the calculation of an LOD value, it does provide a comparison point with which any calculated LOD value could be compared as a quality control on the LOD calculation.

Method C (using the standard deviation of the blank) was not able to be calculated for several analytes due to the absence of signal in the blank samples in the potential region of interest. As such, method C is not suggested as a universal LOD determination method in voltammetric analyses. These results using the blank signal are in support of Benedito and Machado,[23] although in general, the use of blank signals provided a challenge. In contrast, method D, using the standard deviation of the lowest calibrator, has been demonstrated to provide LOD estimations that were consistently within the experimental windows for the analytes tested via cyclic voltammetry and square-wave voltammetry. Therefore, method D may represent a viable approach to LOD determination in voltammetric experiments and could be extended to blanks when they are used as the lowest calibrator and provide a signal. However, caution should be taken as this approach could provide an LOD value that is too low if the replicates are extremely similar in response, as was demonstrated. In these cases, the calculated value should be converted to the signal domain and compared to the noise as a quality control check. In the case where the ASTM E2677-20 method was used as method G, the returned results were comparable to method D, again suggesting that method D represents a good estimate of the LOD in a simple and fast calculation. Additional studies are required, but this may remove the burden of requiring a large number of replicates and possible additional experiments at low levels, which is needed for method G.

Finally, method E (using standard deviation of the y-intercept) and method F (99 % confidence limit) generally provided some of the highest estimations of the LOD. In the case of method E, this method was greatly affected by the regression line and linearity of the data. This may be a useful estimation in some cases where the data suggests a loss of signal that may deviate slightly from linearity in the calibrators, providing an accurate representation of the LOD in less-than-ideal scenarios/electrochemical behavior; however, appropriate statistical methods should be used to minimize these regression challenges first. An example of this was for acetaminophen and naltrexone, where the calibration curves did not approach the origin. In general, method F provided reasonable LOD estimations in the scenarios with the exception of ferrocyanide. It is interesting to note that for the well characterized system of ferrocyanide, the LOD estimation by method F was well outside the experimental range. However, method F provided a conservative LOD estimate in cases that would be related to forensic chemical analysis (gunshot residue and drug detection). Therefore, method F should be used with caution but could provide a conservative LOD estimate. It should be mentioned that in forensic disciplines, depending on how the technique will be implemented (screening/presumptive versus confirmatory) the preference for avoiding false positives or false negatives may change. For example, one may choose to adopt conservative approaches that help to minimize the risk of false positives (minimize the risk of falsely accusing/incarcerating someone). In this case, method F (99 % confidence limit) and method G (ASTM E2677-20) may be desirable from a forensic standpoint in situations or applications where the method provides the final decision, for example when used as a confirmatory method for a situation like workplace drug testing. However, in forensic screening approaches, these methods may not be desirable since the risk of "missing" information would be higher (higher risk of false negatives), for example in presumptive testing to collect evidence or inform confirmatory approaches. It is also worth noting that method F (99 % confidence limit) was also the most mathematically taxing method, and the simpler methods provided results that were similar and in line with the experimental data. **Tables 5 and 6** provide a summary of the electrochemical results.

5. *Conclusions*

Given the vast array of definitions for the limit of detection and the large number of analytical techniques available, the estimation and calculation of the LOD varies between research papers and between techniques. As LOD values are often compared within the literature as an important estimation of the ability of a method or specific technique, researchers should ensure that the LOD values they report are logical and accurate. However, this paper has demonstrated that the estimation of the LOD is not as straightforward as some might think. In the literature for electrochemical sensors, some reported LOD values appear to be either over or underestimated, leading to erroneous conclusions regarding the sensitivity of the method in question or the comparisons to other methods that are being made. As such, this paper has attempted to begin the discussion on voltammetric approaches. It is important to recognize that these results also support the suggestion that any calculated LOD value should be verified experimentally by testing samples spiked at or near the LOD value, something that is not always performed in the literature.

Voltametric sensors are becoming popular in forensic-related research. Since the adoption of novel methods in forensic disciplines typically requires validation and extensive testing, it is fitting to explore approaches for LOD estimation. As has been demonstrated, several methods of estimating the LOD for cyclic voltammetry and square-wave voltammetry resulted in inaccurate reports of

the LOD. In general, the most reliable method of determining the LOD value was utilizing the regression line and the standard deviation of the lowest calibrator. The use of the 99 % confidence limit approach may also present acceptable and conservative estimates. The use of the ASTM E2677-20 method was also demonstrated to be reliable. It is worth noting that both the 99 % confidence limit and the ASTM approach involve a greater degree of effort, however. The 99 % confidence limit approach utilizes a detailed calculation and the use of Student's t-Table, whereas the ASTM method requires ten replicates, at least three calibration levels, and blank samples. On this point, it seems important to reiterate that method D (using the standard deviation of the lowest calibrator) provided comparable and, at times, better estimates of the LOD value with a simpler approach, although in certain scenarios, method F provided the best estimate.

Of the variations tested, the method utilizing the standard deviation of the blank was the least helpful, as many square-wave applications will result in no signal for a blank sample, causing a result of zero. Although the LOD was calculated this way for DPA, it is noteworthy that only half of the replicates presented a signal in the buffer blank. Lastly, the estimation of noise was shown to be beneficial as a comparison point for calculated LOD values, allowing the researcher to compare their calculated value to ensure it would provide a signal above three-times the noise based on the regression (3:1 signal-to-noise ratio).

These results provide a starting point for validation parameter discussions in electrochemistry for forensic purposes. Additional experiments and/or a wider range of electrochemical techniques and analytes should be performed before drawing final conclusions. However, as electrochemistry applications continue to grow in forensic disciplines, research into validation parameters for these methods should be conducted in order to establish consistency between researchers when presenting performance measures. Although a single method may not be viable in every scenario, future studies should seek to determine a conservative approach (which is commonplace in forensic disciplines to avoid false positives) and if a universal method is available, along with the requirement to experimentally test the LOD value. These types of studies are needed to progress the adoption of electrochemistry in forensic analytical schemes and support its use in court.

Table 5. *Summary of the LOD values obtained in the experiments for each method that was tested. LOD units are described in the analyte column and the units for the noise in the signal domain are provided in the noise column. Ferricyanide LOD values are reported as the LOD from the weighted calibration curves.*

Analyte		Method Used to Determine the Limit of Detection (LOD)							
		Noise	Blank	σ_B	σ_{L1}	σ_y	99% C.L.	Serial Dilution	ASTM LOD(95)
		A	B	C	D	E	F	Experimental	G
$[Fe(CN)_6]^{4-/3-}$ (mmol/L)	OX	0.0240 µA	0.001	0.001	0.005	0.001	0.155	0.001 < LOD <0.01	N/A
	RED	0.0090 µA	0.004	N/A	0.003	0.003	0.127		N/A
Lead (µg/mL)	OX	0.0243 µA·V	0.017	0.017	0.021	0.037	0.028	0.02 < LOD <0.2	0.019
Diphenylamine (µg/mL)	OX	0.00006 µA·V	0.282	0.097	0.200	0.370	0.312	≈ 0.2	0.208
Naltrexone (µg/mL)	OX I	0.0006 µA·V	5.980	N/A	0.479	1.098	1.586	LOD < 1	N/A
	OX II		12.92	N/A	0.835	2.111	4.304	3 < LOD < 6	N/A
Acetaminophen (µg/mL)	OX	0.0007 µA·V	0.341	N/A	0.032	0.261	0.112	≈ 0.2	N/A
	RED	0.0030 µA·V	0.381	N/A	0.024	0.232	0.101	0.1 < LOD < 0.2	N/A

OX = oxidation peak, RED = reduction peak, OX I and OX II = oxidation peak I and peak II, respectively.

Table 6. *Summary of the electrochemical potential for each analyte of interest in the study.*

Analyte	Method	Range Tested	Type	Potential / V
$[Fe(CN)_6]^{4-/3-}$	CV	0 mmol/L – 50 mmol/L	OX	+0.281 ± 0.088
			RED	+0.032 ± 0.042
Lead	SWASV	0 µg/mL – 4 µg/mL	OX	-0.777 ± 0.026
Diphenylamine	SWASV	0 µg/mL – 16 µg/mL	OX	+0.434 ± 0.016
Naltrexone	SWV	0 µg/mL – 10 µg/mL	OX I	+0.168 ± 0.010
			OX II	+0.758 ± 0.040
Acetaminophen	SWV	0.1 µg/mL – 100 µg/mL	OX	+0.153 ± 0.008
			RED	+0.198 ± 0.007

CV = cyclic voltammetry, SWV = square-wave voltammetry, SWASV = square-wave anodic stripping voltammetry, OX = oxidation peak, RED = reduction peak, OX I and OX II = oxidation peak I and peak II, respectively.

Conflicts of Interest:

There are no conflicts of interest to declare.

Data Availability:

Associated data can be found at: doi.org/10.18434/mds2-3388

Disclaimer:

Certain commercial products are identified in order to adequately specify the procedure; this does not imply endorsement or recommendation by the National Institute of Standards and Technology (NIST), nor does it imply that such products are necessarily the best available for the purpose.

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