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Simulated enzymatic kinetics in drugs detection



Supervisors:

Prof. Danilo DEMARCHI

Prof. Sandro CARRARA

Dr. Simone AIASSA

Candidate:

Martina VACALEBRE

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*Torino, 31<sup>st</sup> March 2020*

*M.V.*

# Abstract

An emerging field of scientific research is represented by personalized medicine, whose main objective is to adapt the pharmacological therapy for each patient. As a matter of fact, each person presents a different gene pool: consequently, neither standard therapy's effectiveness nor absence of side effects are guaranteed. Therefore, the development of technologies capable of tailoring drugs dosage, especially in cases of combined administration, becomes a key challenge to tackle.

This thesis focuses on drugs detection simulator's implementation. Specifically, by exploiting data obtained by electrochemical experiments and pharmacokinetic parameters reported in literature, a tool capable of generating simulated voltammograms is proposed. Thanks to machine learning, these voltammograms are the starting point to build synthetic dataset aimed at developing new P450-based electrochemical sensors. The final goal is the prediction of therapeutic doses in the blood of oncological patients for precision medicine.

The proposed research deals with multi-drugs monitoring for personalized therapy. In 2011, Prof. Sandro Carrara established a theory about drug interactions on electrochemical sensors. Based on the proposed mathematical model, an algorithm able to reproduce the interactions between different drugs with distinct kinetics is developed. These interactions represent the main hurdle for the development of P450-based electrochemical sensors in exogenous metabolites' detection. In fact, included interferences in obtained signals turn out to be complicated to elaborate.

To ease the use of the algorithm, a graphical user interface (GUI) was designed using both software MATLAB® and Python. Moreover, a connection with a database MySQL® was set up: data related to drugs used in the simulations are recorded in it.

Different combinations of drugs were simulated: cyclophosphamide/dextromethorphan, to evaluate the enzymatic-inhibition case, and naproxen/flurbiprofen, for the enzymatic-activation case. In addition to the mentioned drugs, other seven drugs with related data are recorded in the database.

Moreover, undertaken simulations highlighted some imperfections in the scientific paper of reference. Thus, the implementation of the theory and the revision of the proposed equations were possible.

In conclusion, this work can be considered as a starting point for the development of a new electrochemical sensors' generation for exogenous metabolites. Obtained data from the proposed simulator, united with artificial intelligence, could result in an efficient approach to overcome actual challenges in the realization of devices for drugs monitoring in personalized therapy.

# Sommario

Un emergente filone della ricerca scientifica è rappresentato dalla medicina personalizzata, il cui principale obiettivo è adattare la terapia farmacologica per ogni singolo paziente. È infatti noto che, in base al proprio corredo genetico, ogni individuo presenta specificità uniche, tali per cui non sempre è garantita l'efficacia di una cura standardizzata o l'assenza di effetti collaterali per una stessa dose di farmaco. Di conseguenza, è auspicabile identificare delle tecnologie capaci di personalizzare il dosaggio dei farmaci, in particolar modo nei casi di somministrazione in combinazione.

Questa tesi si focalizza sull'implementazione di un simulatore di rilevamento di farmaci. In particolare, ricorrendo ai dati ottenuti da esperimenti elettrochimici e ai parametri farmacocinetici presenti in letteratura, si propone un tool capace di generare voltammogrammi simulati. Questi ultimi sono il punto di partenza per la costruzione di dataset artificiali volti a sviluppare, con l'aiuto dell'apprendimento automatico (noto come *machine learning*), nuovi sensori elettrochimici basati su citocromi P450. Lo scopo finale di questi sistemi è la predizione delle dosi corrette di farmaci chemioterapici per applicazioni nella medicina personalizzata.

La ricerca svolta e proposta in questo lavoro tratta il rilevamento simultaneo di farmaci su sensori elettrochimici. Basandosi sulla teoria proposta dal Prof. Carrara nel 2011, è stato sviluppato un algoritmo capace di riprodurre le interazioni tra farmaci con diverse cinetiche enzimatiche. Queste interazioni rappresentano l'ostacolo principale alla realizzazione di sistemi elettrochimici che sfruttino le potenzialità di citocromi P450 per il rilevamento di metaboliti esogeni, in quanto le interferenze presenti nei segnali ottenuti risultano complicate da elaborare.

Per rendere agevole l'utilizzo dell'algoritmo elaborato, usando i software MATLAB® e Python, sono stati predisposti un'interfaccia utente (GUI, graphical user interface) e un collegamento a un database MySQL®, in cui sono registrati i dati relativi ai farmaci necessari alle simulazioni.

Sono state simulate le combinazioni di farmaci ciclofosfamide/destrometorfano, per valutare il caso di inibizione enzimatica, e successivamente, la coppia naprossene/flurbiprofene per il caso di attivazione enzimatica. Oltre i farmaci nominati, altri sette farmaci con i relativi dati sono presenti nel database a disposizione dell'utente.

Inoltre, le simulazioni effettuate hanno permesso di rilevare alcune imperfezioni nell'articolo scientifico di riferimento, consentendo l'implementazione della teoria e la revisione delle equazioni proposte.

In conclusione, questo lavoro può essere considerato come un punto di partenza per lo sviluppo di una nuova generazione di sensori elettrochimici per metaboliti esogeni. I dati ottenuti dal simulatore proposto, uniti all'impiego dell'intelligenza artificiale, potrebbero

risultare un approccio efficace per superare gli ostacoli attuali nella realizzazione di questi sistemi.

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# 1.Introduction

The aim of this work is about building a drugs detection simulator. The idea consists in exploiting data obtained by electrochemical experiments and pharmacokinetic parameters present in literature to realize simulated voltammograms. These voltammograms are useful to obtain a synthetic dataset for a new point-of-care system based on machine learning and cytochrome P450-based electrochemical sensors. Thanks to artificial intelligence, the final goal is the prediction of therapeutic doses in the blood of oncological patients, for applications to precision medicine.

Currently, cytochrome P450-based electrochemical sensors for drugs detection don't find a systematic application in real practice. Although their high potential in personalized treatments, their development is still hindered by many hurdles, such unsatisfying elaboration data and lack of standardization methods.

Different drugs could be simultaneously present in the patient's blood and some of them could be catalysed by the same cytochrome isoform. The real metabolite's concentration could be then hidden by other drugs' signals.

As student in exchange at EPFL in Lausanne, I carried out a research about multi-drugs monitoring for personalized therapy and this thesis sums up the results. In 2011, Prof. Sandro Carrara established a theory about drug interactions on electrochemical sensors. Based on the proposed mathematical model, an algorithm capable to reproduce the interference between different drugs is developed in this thesis. The main challenge concerns the simulation of different combinations of drugs, each one following a certain kinetics. Moreover, simulations need to account for experimental variability, which usually affect reproducibility as well.

As mentioned above, data related to specific drugs are founded in literature and recorded in a database. Future extension of the database with other drugs are then possible.

This thesis deals with the complex data's issue resulting from using drugs in combination (i.e. cyclophosphamide/dextromethorphan, naproxen/flurbiprofen) and an algorithm for the simulation of cross-correlated drugs' voltammograms is then developed using both MATLAB® and Python.

A particular emphasis will be reserved to the comparison of the experimental results found in literature with data obtained by simulated drugs detection using both software. The similarity between them will validate the simulator's work.

Final remarks are devoted to challenges and to opportunity to translate the simulated data in clinical practice.

## 2.State-of-the-art

### 2.1 Metabolites

Firstly, it is important to describe the target molecules of the simulator. A metabolite is defined as a substance involved in metabolic processes in an organism. It could be a product of a reaction or a molecule that allows the reaction to occur. Examples of metabolites are glucose, cholesterol, lactate and glutamate, common compounds in the bloodstream. Since metabolism has a key-role in the energy-related regulatory functions in the living beings, the detection of metabolites can provide a large class of useful data for diagnosis and therapy.

A distinction between endogenous and exogenous metabolites is necessary: the first ones are internally produced by cells; the second ones are provided from outside [1]. For instance, all the drugs used in the health-care therapies are exogenous compounds. In some cases, this concept is relative as some metabolites can be classified in both categories: insulin is a hormone secreted in the pancreas, but it can also be injected in diabetic patients.

### 2.2 Electrochemical sensors

A suitable solution to detect metabolites is electrochemical sensors. It is due to their fast, accurate and quantitative detection together with their easy and cheap usability [2].

Electrochemical detection is based on electrical sensing thanks to redox reactions. In a redox reaction, two chemical half-reactions occur: an oxidation, where an atom loses electrons, and a reduction, where an atom gains them. Consequently, this exchange of charged particles generates a current, that can be measured if a proper system of electrodes is used. The measured current is strictly linked to the concentration of the chemical species involved.

In this thesis, voltammetric techniques are deeply investigated. In voltammetry, the current that occurs on a working electrode in an electrochemical environment is measured, while the potential that is applied is varying [3]. As shown in Figure 1, a three-electrode set-up is used to carry out this measure: a working electrode (WE), that can be functionalized to increase the sensitivity or the specificity of the system, a reference electrode (RE), to apply the correct voltage across the electrochemical cell, and a counter electrode (CE), to measure the current due to the redox reaction. The electric circuit that controls the applied potential and collects the measured current is called *potentiostat* [4].

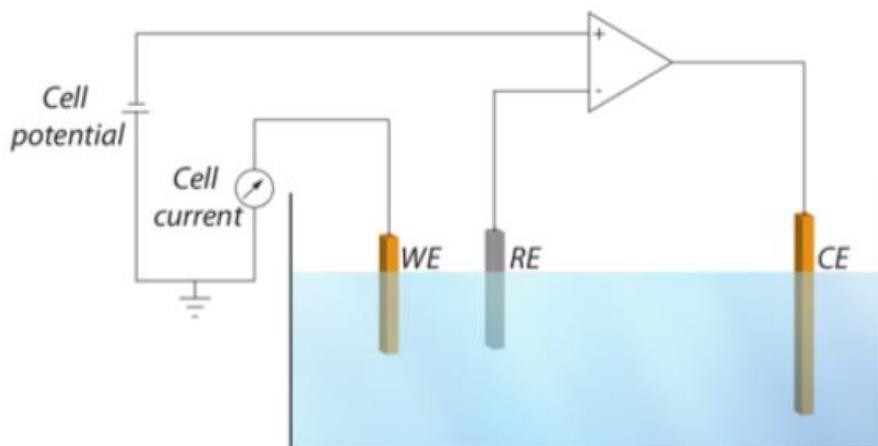


Figure 1. Three-electrode set-up with potentiostat. Reprinted from [4].

## 2.3 Voltammograms

A versatile technique in the voltammetric domain is cyclic voltammetry (CV). It is obtained by collecting continuously the current at the working electrode during a potential sweep. As shown in Figure 2.a, the applied voltage has a triangular waveform because it is reversed when it reaches a specific value. In the case of fully reversible process, the final plot, called *voltammogram*, will show the measured current as a function of the applied voltage, obtaining a typical shape as illustrated in Figure 2.b.

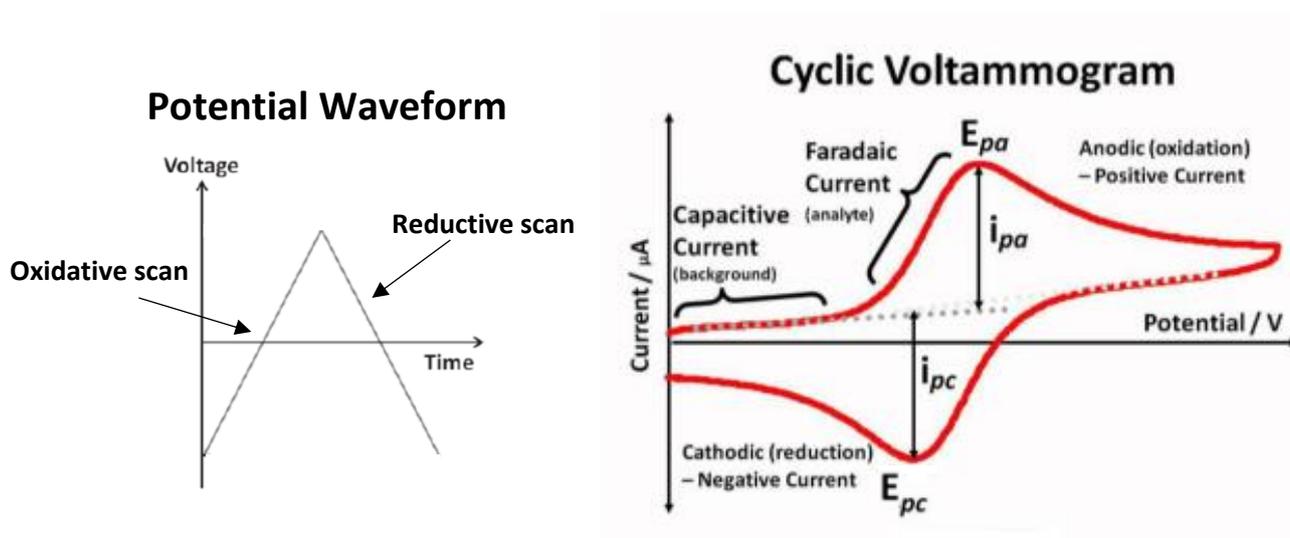


Figure 2.(a) A potential sweep. (b) Resulting voltammogram using IUPAC convention.

Firstly, the potential range to scan is opportunely decided and set. During the forward scan, the potential increases toward positive potentials with a constant speed, called *scan rate* ( $v$ ), and a positive current peak is recorded. In greater detail, redox species in solution accept

electrons from the working electrode, thus oxidation occurs. After reaching the turning point, the potential is ramped in the opposite direction to return to the initial value [5]. A negative current peak due to the reduction of the redox species in the solution is pointed out.

As shown in Figure 2.b, initially the output is a capacitive current due to the background: electrical charges in the solution accumulate on the surface of the working electrode and no chemical reaction is involved. On the other hand, faradaic currents originate from redox reactions on the electrode surface [6].

The usefulness of this analysis is related to the possibility to see at which potential electrochemical reactions occur and for the determination of electroactive species present in a solution [1]. In fact, the two peaks in the graph corresponds on the x-axis to the oxidation and reduction potentials ( $E_{PA}$  and  $E_{PC}$ ). In particular, the half-difference between them represents the *standard potential* and it depends on which reference electrode is used in the experiment. On the contrary, the difference between the standard potential and the oxidation one doesn't change, because it is exactly the voltage required by the redox reaction.

Cyclic voltammetry is used also as a quantitative analysis to determinate the concentration of the redox species. According to Randles–Sevcik equation, the current peak is proportional to the concentration of the reagent.

$$i_p = 0.4463nFAC \left( \frac{nFvD}{RT} \right)^{1/2} \quad (1)$$

In Equation 1, A is the electrochemical active area of the working electrode (in  $\text{cm}^2$ ), v is the scan rate (in  $\text{mV/s}$ ), n is the number of electrons involved in the redox reaction and D is the diffusion coefficient (in  $\text{cm}^2/\text{s}$ ). A modified version of Randles - Sevcik equation is used as starting point to simulate voltammograms in the tool described in this thesis [7].

## 2.4 Characterization of electrochemical sensors

Electrochemical sensors are characterized by two different parameters: sensitivity and limit of detection (LOD). Sensitivity corresponds to the slope of calibration curve, a graph where the Faradaic current is a function of metabolite's concentration, as shown in Figure 3 in case of hydrogen peroxide detection [8]. In literature, the value of sensitivity can be expressed in  $\mu\text{A}/\mu\text{M}$  or normalized by electrochemical active area of the electrode (Equation 2) and expressed in  $\mu\text{A}/\mu\text{M} \cdot \text{mm}^2$  [8].

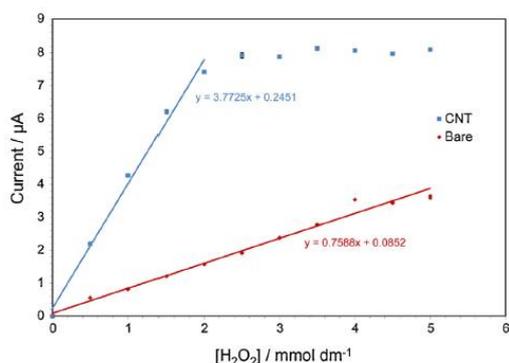


Figure 3. Calibration curve for hydrogen peroxide detection. Reprinted from [8].

$$S = \frac{\Delta I}{A * \Delta C} \quad (2)$$

Carrara *et al.* already demonstrated the possibility to enhance the sensitivity by modifying the surface of the electrode with nanostructures, i.e. multiwalled carbon nanotubes [8]. Mainly, their role is to increase the electrochemical active area and, as a consequence, the sensitivity. Moreover, they demonstrated that the constant part of Randles – Sevcik equation (Equation 1), can be replaced by the sensitivity [8].

$$S = \frac{nF(A_g + A_{CNT})}{A_g} \sqrt{\frac{nFD\nu}{RT}} \quad (3)$$

Instead, the limit of detection (LOD) is the minimum amount of drug that the electrode can detect and is calculated in accordance with equation 4, where S is the sensitivity,  $\delta i$  is the standard deviation of blank current measurement and  $\sigma$  represents the confidence interval [8].

$$LOD = \sigma \frac{\delta i}{S} \quad (4)$$

$$\sigma = \begin{cases} 1 \rightarrow 68.3\% \\ 2 \rightarrow 95.4\% \\ 3 \rightarrow 99.7\% \end{cases} \text{ confidence interval.}$$

## 2.5 Cytochromes P450

Cytochromes P450 (CYPs) are a family of enzymes characterized by the presence of a heme group as cofactor [9]. They are responsible for the transformation of over 200,000 chemicals, including several exogenous and endogenous compounds [9]. In the human body, they are localized in the hepatocytes since they have a key role in the metabolism: 57 CYPs were identified in humans and 15 of these are necessary to metabolize drugs and other species [10].

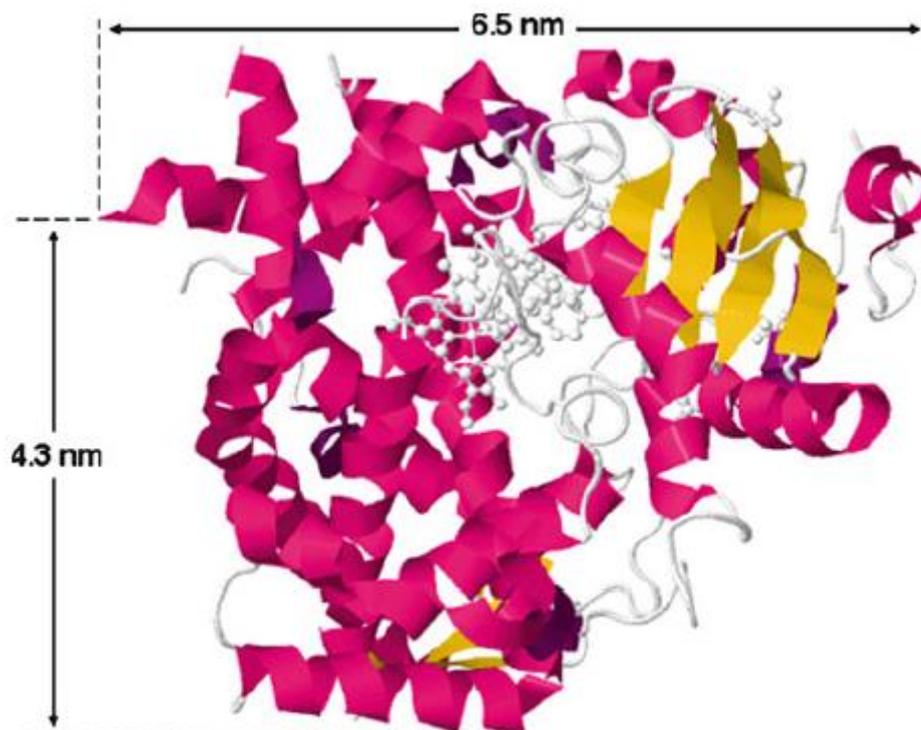
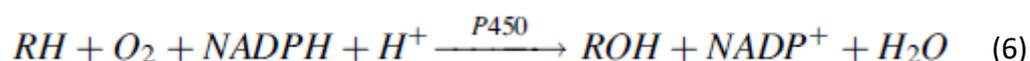


Figure 4. Crystallographic structure of cytochrome P450 3A4. Reprinted from [1].

As a matter of fact, CYPs are redox enzymes, biological molecules that catalyse redox reactions and involve transfer of electrons between chemical species. This is due to the presence of iron in the heme core: it can change its state of oxidation as shown in Equation 5 [9].



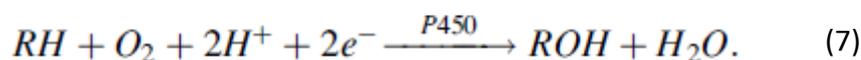
In detail, the metabolism of drugs by CYPs involves an oxygen molecule and an electron donor [11]. As in all anabolic reactions in human body, nicotinamide adenine dinucleotide phosphate (NADPH), which is an oxidoreductive coenzyme, plays the role of electron donor. The hydroxylation reaction is as follows in Equation 6 [1]:



The role of the cytochromes P450 is to immobilize the drug molecule and to oxidize it thanks to the reduction of the oxygen [1]. NADPH switches in its oxidized form.

Thanks to these important ability, cytochromes P450 are of great interest for the development of electrochemical biosensors both for detection and monitoring of

metabolites [10]. In this case, necessary electrons for the redox reaction are provided by the electrode, resulting in a current strictly linked to the activity of the cytochrome immobilized on the electrode surface. Equation 7 sums up what happens in the proximity of the electrode [1]:



Consequently, it is possible to carry out an amperometric detection of metabolites and to obtain the related voltammograms. Moreover, it is possible to enhance the sensibility of the detection system using multiwalled carbon nanotubes (MWCNTs), often exploited as electron transfer mediators [8]. This is mainly due to the fact that they increase the electrochemical active area, important parameter taken in account also in Randles-Sevcik equation (Equation 1). In fact, if the electrical active area increases, there will be more available surface for redox reactions.

The CV of a CYP without drugs will not show oxidation peaks, because the reaction turns out to be as a reduction. This CYP's current peak could be overlapped by peaks due to drugs detection.

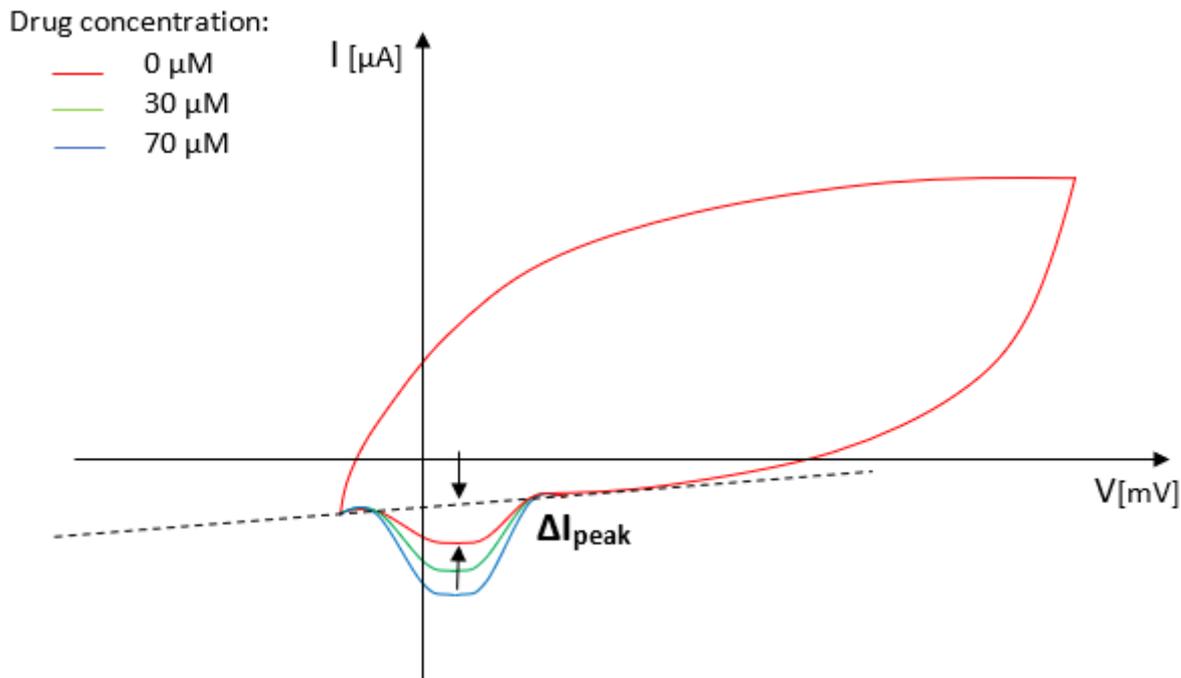


Figure 5. Example of P450 voltammogram with and without the presence of drug.

The graph ends up being quite difficult to interpret and it requires manual post-processing using analytical techniques. This is one of the reasons why CYPs-based electrochemical

biosensors for exogenous metabolites are not still present on the market: it is necessary a system capable to elaborate this complex data.

## 2.6 Enzymatic kinetics and interferences

Several metabolites can be catalysed by the same cytochrome P450 and with different enzymatic kinetics. Commonly, it is assumed that Michaelis-Menten kinetics is the model that substrates follow when singly involved in their biochemical transformations. Michaelis-Menten equation (Equation 8) expresses a kind of interaction between enzyme and substrate:  $K_M$  is the substrate concentration when the rate of the reaction assumes the half-maximum value [1]. Thus,  $K_M$ , called *Michaelis' constant*, is linked to the enzyme's efficiency.

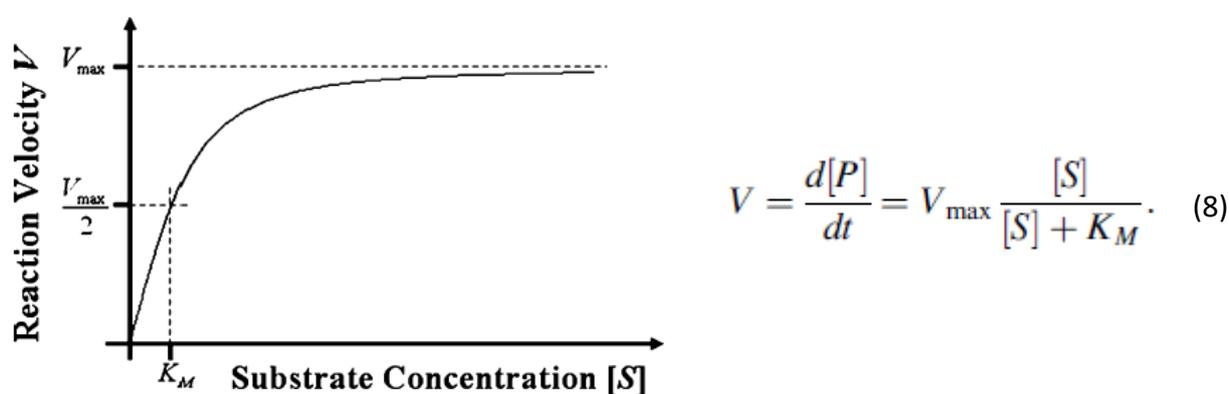


Figure 6. Reaction velocity vs substrate concentration. Reprinted from [1].

On the other hand, CYPs can catalyse the oxidation of substrates also by atypical kinetics as biphasic (Figure 7.c), sigmoidal (Figure 7.a) or substrate-inhibition kinetics (Figure 7.b) [1]. This is due to the fact that some enzymes are not selective and have several substrates. Biphasic reactions are characterized by an initial resembling Michaelis-Menten profile, but without reaching an asymptotic level [12]. On the contrary, sigmoidal reactions have a very low rate until the substrate reaches a higher concentration, ending with an asymptotic profile [1]. In substrate inhibition case, a higher concentration of substrate inhibits enzyme's functions, resulting in a decrease of the reaction rate [1].

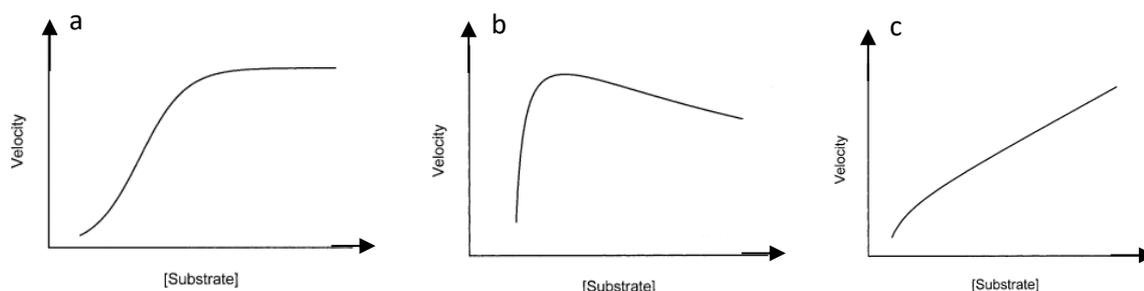


Figure 7. Atypical kinetics profiles. a) Sigmoidal profile. b) Substrate inhibition profile. c) Biphasic profile. Adapted from [12].

If instead of considering a single substrate, two different compounds that react on the same CYP are taken into account, the issue becomes more complicated. In fact, the simultaneous co-presence will result in an interference. Particularly, the conformation of the enzyme can change and so its efficiency for a specific substrate can increase or decrease. Mainly, for the purpose of this thesis, there are two cases of interaction: hetero-activation and inhibition. In the first case, an enzyme could require an activating substrate 1 to catalyze a substrate 2. On the contrary, inhibition can be either competitive or non-competitive: in the first, two different substrates suit for the same active site of the enzyme, whereas in non-competitive inhibition the bond of a substrate 1 with the enzyme impedes the catalysis of substrate 2. Specifically, a competitive inhibitor acts at the catalytic site of the enzyme, whereas an allosteric modulator (inhibitor or activator) binds a not catalytic site (allosteric site). An enzyme can possess several allosteric sites that recognize several inhibitors/activators.

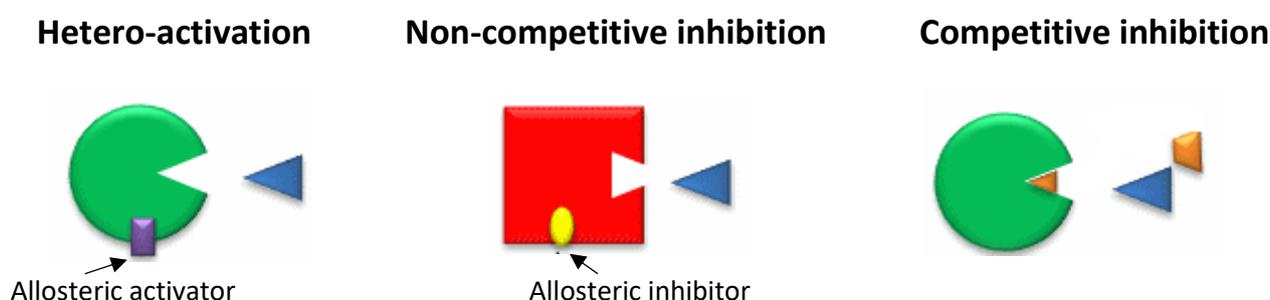


Figure 8. Examples of substrate-enzyme interactions. Adapted from [30].

## 2.7 Multi-detection by single cytochrome P450

This thesis starts from the paper written by Carrara *et al.* entitled "Multi-panel drugs detection in human serum for personalized therapy" [7]. In this article, they propose a new theory to discriminate different contributions given by drugs in voltammetric detection [7]. It is based on the approximation of voltammograms to Gaussian curves: this generalization fairly works with faradaic process, but doesn't consider the diffusion phenomena, that mostly emerges after the current peak.

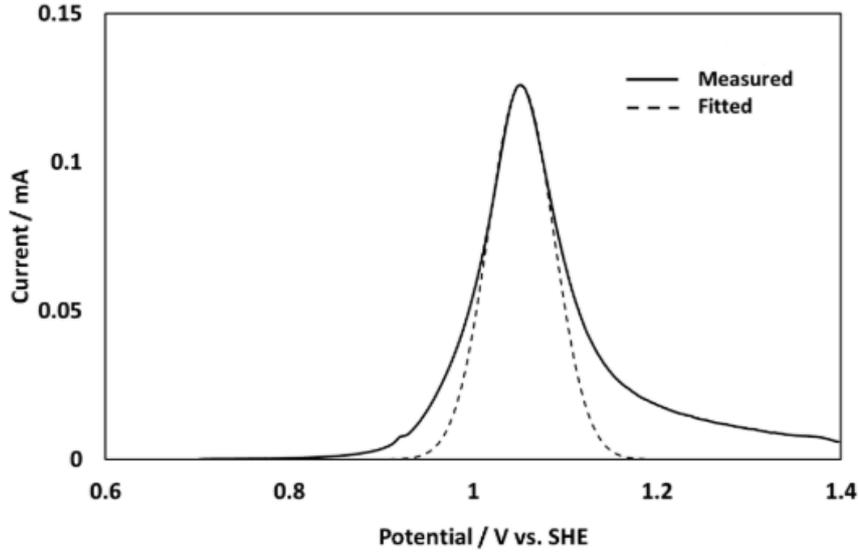


Figure 9. Example of voltammogram fitted with Gaussian function. Adapted from [31].

Nevertheless, Gaussian approximation is considered a good trade-off. In the paper, the different contributions due to the simultaneous co-presence of drugs are summed as Gaussian curves to obtain the total current, as shown in Equation 9.

$$i(V) = \sum_{\forall k} \Gamma_k C_k \left\{ \prod_{\forall j \neq k} A_k(C_j) \right\} e^{-\frac{(V-V_k)^2}{\sigma_k^2}} \quad (9)$$

$$\Gamma_k = 0.4463 \left( \frac{n^3 F^3 \nu D_k}{RT} \right)^{1/2} A \quad (10)$$

It is necessary to highlight that Equation 9 and its constant parameter (Equation 10) reminds Randles-Sevcik equation (Equation 1), but it takes into account the possible interaction with drugs. In fact, in case of enzymatic-activation, the current peak's amplitude of k-drug  $A_k$  will increase depending on the concentration of j-drug  $C_j$  [7]. In the same way, in case of enzymatic-inhibition, it will be noticed a reduction of the peak amplitude [7].

<b><i>Inhibition case</i></b>	<b><i>Activation case</i></b>
$A_k(C_j) = A_{k0} (1 - \varepsilon_j w_j)$	$A_k(C_j) = A_{k0} (1 + \varepsilon_j w_j)$

(11)

$\varepsilon_j$  is a parameter linked to the interference strength between the different metabolites and can assume values between zero and one [7].  $w_j$  is the velocity of the enzymatic reaction

and depends on the concentration of the interfering substrate. In the case of Michaelis-Menten kinetics, it is shown in Equation 12.

$$w_j = \frac{V_{\max} C_j}{K_M + C_j} \quad (12)$$

### 3. Materials and methods

In this chapter, the simulator’s graphical user interface (GUI) designed on MATLAB® (version R2016a) and the related drugs’ database managed on MySQL® will be described. Successively, the algorithm developed on MATLAB® will be introduced in greater detail.

#### 3.1 Graphical user interface (GUI)

It is commonly known that a graphical user interface (GUI) should be user-friendly, easy on the eyes, flexible and effortlessly provides different paths to reach the desired function. The contents must be organized and structured in order to ensure the greatest accessibility to the information while avoiding any kind of ambiguity.

Following these principles, the simulator’s GUI is designed as shown in Figure 10. An extract of the main GUI’s code is reported in Appendix (A.1).

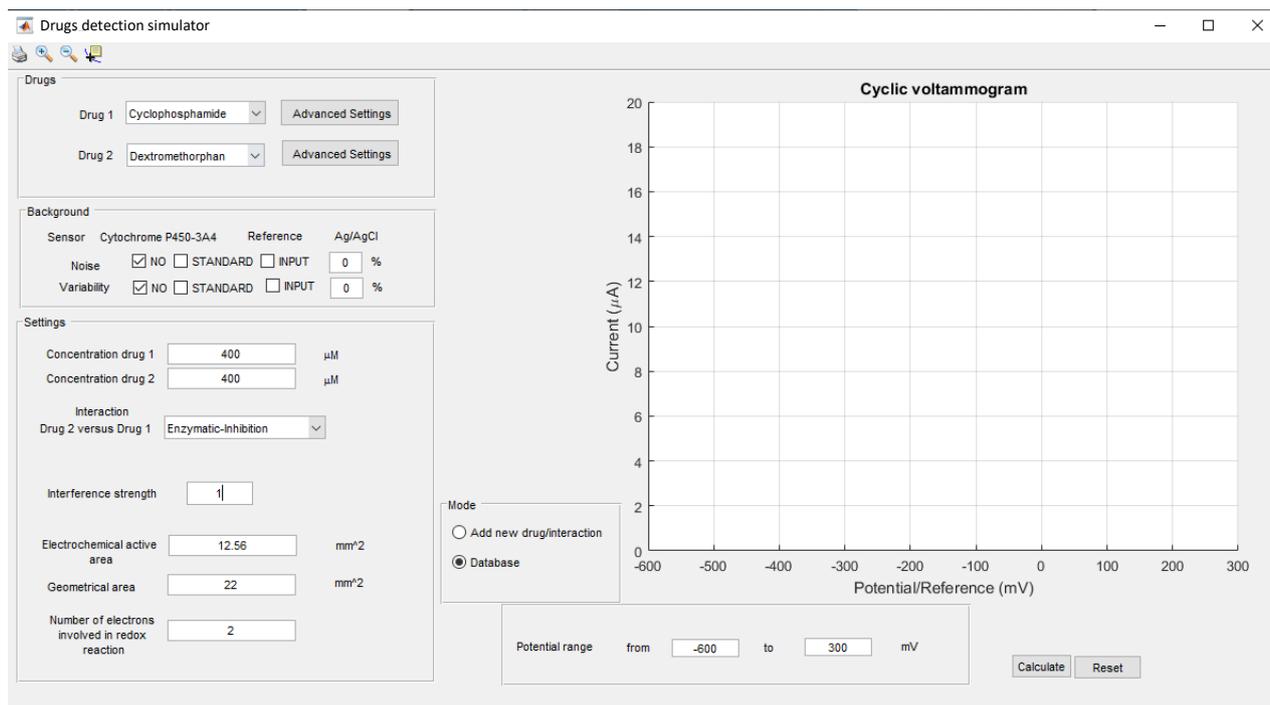


Figure 10. Graphical user interface of the simulator.

Different panels are used to organize the interface:

- “Drugs”: the user can see the chosen drugs and the concerning features;
- “Background”: information related to the simulation’s context are summed up;
- “Settings”: the user can set up the specific characteristics related to the simulated experiment;

- “*Mode*”: the user can decide either to work with drugs already present in the database or to create a new record in the database and use a new drug.

### 3.1.1 Drugs

If the button “*Advanced settings*” is pressed, data concerning the chosen drugs and collected from the database will be shown in an additional GUI as in Figure 11. Especially, the user will see the voltammogram’s features, like peak’s position and peak’s width, and the pharmacokinetics’ parameters. It is possible to customize the simulation by changing these settings. The “*Default*” button cancels all modification and resets the parameters to the original records from the database.

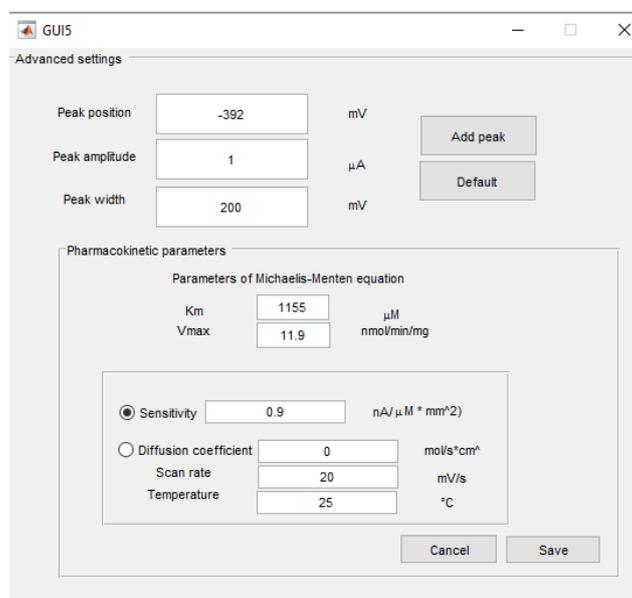


Figure 11. *Advanced settings* GUI.

As already mentioned above, sensitivity can replace the constant part in Randles – Sevcik equation (Equations 1 and 3). Thus, in the “*Advanced settings*” panel, the user can decide either to use sensitivity or the original parameters present in the formula. Sensitivity’s values are obtained from literature and recorded in the database for each drug.

### 3.1.2 Background

This panel is used to set “*noise*” and “*variability*”: in the first feature, it is considered both chemical and electrical noise; in the second one, the Brownian behaviour of voltammograms is taken into account. In reality, electronic noise is due to the measurement system and to the mains frequency. Commonly, electronic noise (Equation 13) includes Johnson–Nyquist thermal noise (Equation 14), which is temperature-dependent, shot noise, due to the discrete nature of electric charges, and flicker noise (Equation 15), which is the main noise source for low frequencies [13]. Shot noise is independent from frequency and temperature, thus it is considered white noise together with thermal noise [13]. On the contrary, flicker noise has a spectral density [13].

$$I_n = I_{thermal} + I_{shot} + I_{flicker} \quad (13)$$

$$I_{thermal} \rightarrow PSD = \frac{4kT}{R} \quad (14)$$

$$I_{flicker} = \frac{A}{f} \quad (15)$$

Actually, the chemical noise has a greater impact on the measurements than the electrical one. Chemical noise linearly increases with the chemical compound's concentration. It is evident in chronoamperometry, an electrochemical technique where the current collected at the working electrode at a fixed potential is plotted as a function of time. It is supposed that during the measurement's time, the concentration of the detected compound increases: in the case of a lab experiment, there will be further injections; in the case of chronoamperometric detection-based devices, the metabolites' concentrations will change in the bloodstream over time. An example is shown in Figure 12.

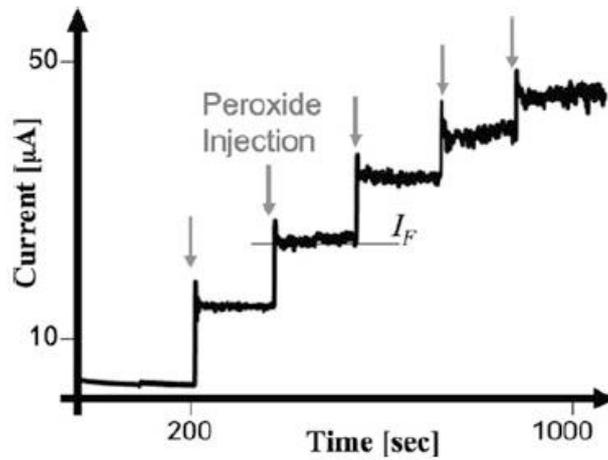


Figure 12. Example of chronoamperometry. Reprinted from [1].

In the simulator, the user can decide either to ignore the noise, choosing the checkbox "NO", or to consider it, choosing one of the two checkboxes "STANDARD" or "INPUT": if "STANDARD" noise is selected, an average percentage equal to 2,5 % will be added to the current as white gaussian noise (WGN); if the user prefers to decide which percentage to add as WGN, "INPUT" can be selected and the chosen value can be written in the dedicated text box. In any case, the function "noise" will evaluate the user's choice and assign the right value to the variable "noise": it will take into account the initial offset due to the noise, on average around 1 µA, and will add the desired percentage (%Noise; it is equal to 2,5% in the standard case) as shown in Equation 16. As mentioned above, a direct proportionality exists between species' concentration and noise  $I_N$ : in equation 16, the variable  $x$  represents the

concentration. Equation 16 is an empirical formula based on evidence, no data about it was found in literature. Finally, the calculated value  $I_N$  will be added to the simulated currents.

$$I_N(x) = I_{offset} + \frac{\%Noise}{100} * I_{TOT}(x) \quad (16)$$

An example of simulated voltammogram with addition of noise “STANDARD” is shown in Figure 13.b.

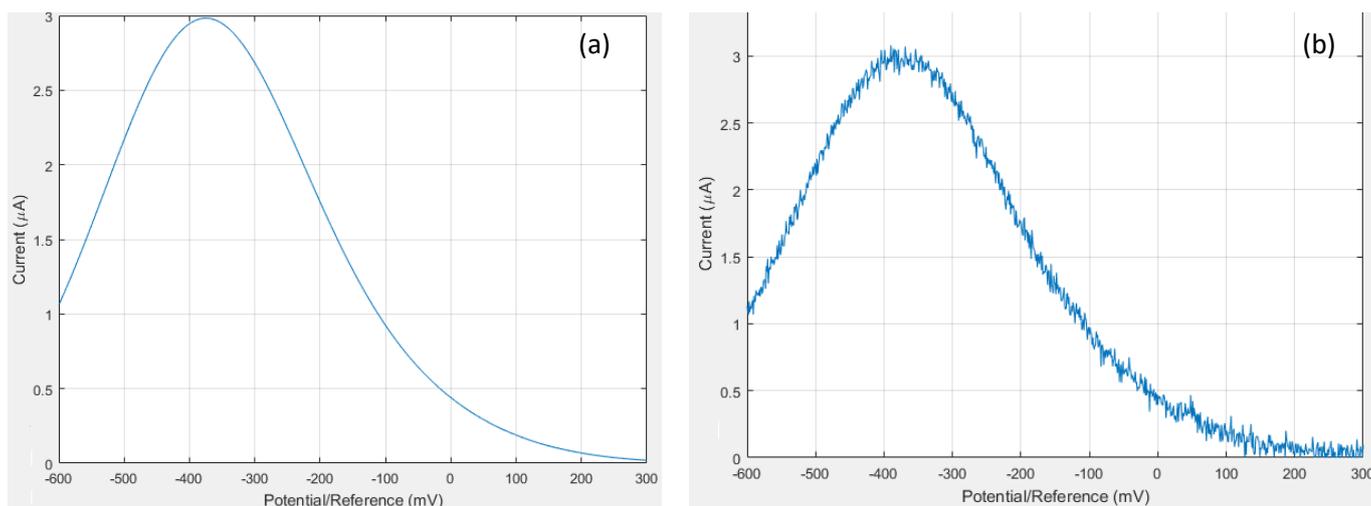


Figure 13. Example of voltammogram's simulation (a) without and (b) with noise addition.

The second parameter in “Background” panel is “variability”; in reality, two voltammograms of the same species in the same conditions will be similar but not the same. “Variability” checkboxes work in the same way as “noise” ones: in the “STANDARD” option, a variation equal to  $\pm 10\%$  is considered in the peaks’ positions and amplitudes; in “INPUT” mode, the user can decide the variability’s percentage. The function “variability” will randomly assign to the variable “variability” a percentage included in the considered range and will modify peak’s features. The function “variability” is reported below in Appendix (A.4).

### 3.1.3 Settings

The first parameters to set in the “Settings” panel are drugs’ concentrations. The user will choose which simulated interaction occurs thanks to a pop-up menu: “Enzymatic-Activation”, “Enzymatic-Inhibition” and “No interference” are the possible choices. It is specified that the simulation will show the influence that Drug2 will have on Drug1 (Drug2 vs. Drug1). For instance, in a case of enzymatic-inhibition, dextromethorphan (DX) will interfere with the detection of cyclophosphamide (CP) if cytochrome P450 3A4 is used [7]. Both drugs can be catalysed by this enzyme and the presence of dextromethorphan inhibits the enzymatic activity necessary for the interaction cyclophosphamide-cytochrome. If this

case is simulated, dextromethorphan should be considered as Drug 2 and cyclophosphamide as Drug1.

In the main code, using an “if/elseif” statement, a loop is implemented to read the desired inference and to recall the specific function among “*activation*”, “*inhibition*” and “*interaction*”. In these functions, the simulated voltammograms are generated following the Equations 9-12. The function “*activation*” is reported below in Appendix as example (A.3).

The user can also repeat the same simulation with different values of interference strength. In fact, this new parameter was introduced by Carrara *et al.* and no data is available in literature [7]. In order to optimize this parameter, the user can carry out several simulations until the value that provides the most realistic output is found.

The last parameters to set are geometrical area and electrochemical active area of the electrode (in mm<sup>2</sup>) and number of electrons involved in the redox reaction. Finally, the user can decide the potential range to scan, considering that the resolution will be 1 mV.

#### 3.1.4 Mode

If “*Database*” is selected in the “*Mode*” panel, it is possible to choose the two drugs whose interaction will be simulated thanks to two pop-up menus (also called *context menus*). The menu will show the drugs already recorded in the database (DB) and the related data will be called back from it. In fact, a function called “*readdatabase*” makes a connection to the DB using an ODBC (Open Database Connectivity) driver and assigns the values to the output variables. Finally, it closes the connection before proceeding with the main code. The function “*readdatabase*” is reported below in Appendix (A.2).

The values of the variables in the MATLAB® workspace are extracted and saved in the local variables thanks to the function “*interaction\_data*”.

#### 3.1.4 Calculate

When all the parameters are configured, the button “*Calculate*” should be pressed to run the simulation. “*Calculate\_callback*” function contains all the instructions to read the inputs in the main GUI, elaborate data and show the resulting simulated voltammogram in the appropriate graph space on the right. “*Calculate\_callback*” is reported in Appendix (A.1).

A button “*Reset*” was designed to refresh the GUI and restart the simulation.

### 3.2 Database

A list of drugs and the related parameters are recorded in a database on MySQL®. Essentially, drugs investigated in previous studies and with accessible data are chosen to create a first basic database, consisting of eleven records and reported in Table 1. They belong to different pharmacological categories: four chemotherapeutic agents, two anti-inflammatories, one

cough suppressant, one anti-obesity drug, two anaesthetics and a fever-reducing painkiller. In reality, it could be possible to find mixed presence of these drugs in the patient's blood.

Table 1

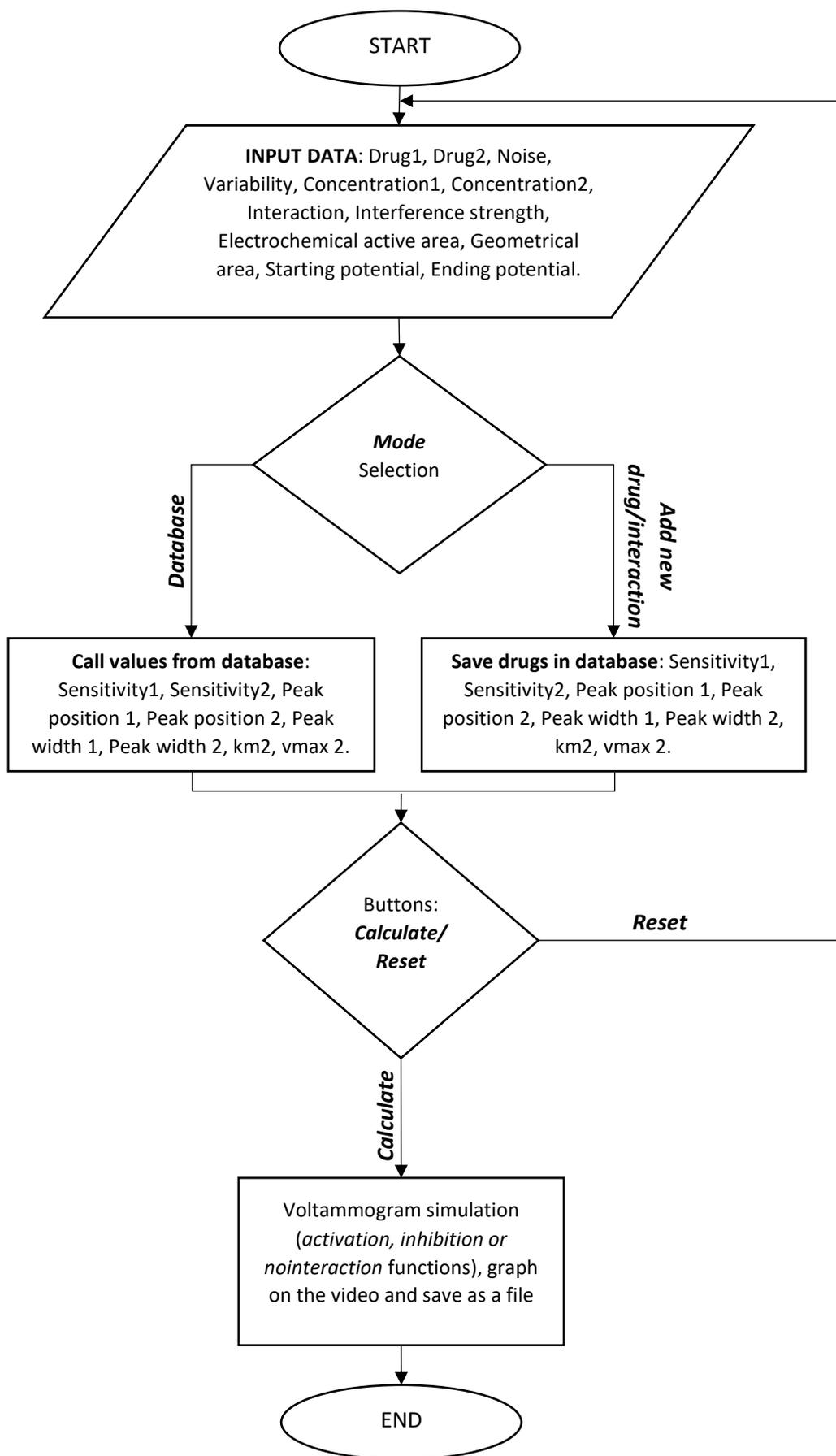
Drug	Sensitivity [nA/ $\mu$ M*mm <sup>2</sup> ]	Peak position [mV]	Peak width [mV]	$k_m$ [ $\mu$ M]	$V_{max}$ [nmol/min/mg]	Reference
Cyclophosphamide	0.63	-296	100	7000	2.3	[7], [14]
Dextromethorphan	6.63	-392	200	1155	11.9	[7], [15], [16]
Flurbiprofen	0.46	60	200	1.9	343	[7], [17]
Naproxen	0.25	-10	200	143	0.84	[7], [18]
Propofol	0.32	600	200	18	2.6	[19], [20]
Paracetamol	2.46	700	400	474	0.79	[19], [21]
Midazolam	48.8	-700	200	7.46	0.696	[22], [23]
Etoposide	9.1	220	150	77.7	314	[24], [25]
Ifosfamide	0.4	-450	150	8.1	1.2	[24], [14]
Ftorafur	3.9	-450	150	15	0.13	[24], [26]
Benzphetamine	20.5	-200	150	240	3.9	[7], [27], [28]

Future improvements of the tool should be addressed to extend this list, by investigating other drugs that could interact on a sensor. Data already present in literature is not exhaustive: same drugs in different conditions, like solution or pH, present different features. Data reported in DB can be considered a good approximation and an encouraging starting point.

### 3.3 Algorithm structure

In figure 14, a flowchart shows algorithm's structure. It highlights the choices the user can do and input/output data. Some extracts of the tool's code are reported in Appendix.

Figure 14. Flowchart algorithm.



# 4. Results and discussion

In order to verify the correct behaviour of the algorithm, some simulations are carried out and shown in the next paragraphs. It is necessary to clarify that Equations 9-12 are the basis of the algorithm and they have never been tested before. Finally, progress made to the theory thanks to the simulator will be discussed.

## 4.1 Simulations

### 4.1.1 Enzymatic-Activation

Firstly, the algorithm to simulate a voltammogram in case of enzymatic-activation on a P450-based multiple detection is evaluated. The goal is to confirm the capability of the tool to increase the current peak related to a Drug1 when Drug2's concentration increases whereas Drug1's concentration is constant. Thus, two simulations are carried out in the same conditions, by varying only Drug2's concentration. Parameters of the first test are summarised in Table 2.

Table 2

	Simulation 1	Simulation 2
C1 [ $\mu\text{M}$ ]	500	500
C2 [ $\mu\text{M}$ ]	500	800
Position1 [mV]	200	200
Position2 [mV]	600	600
$\epsilon$	0.5	

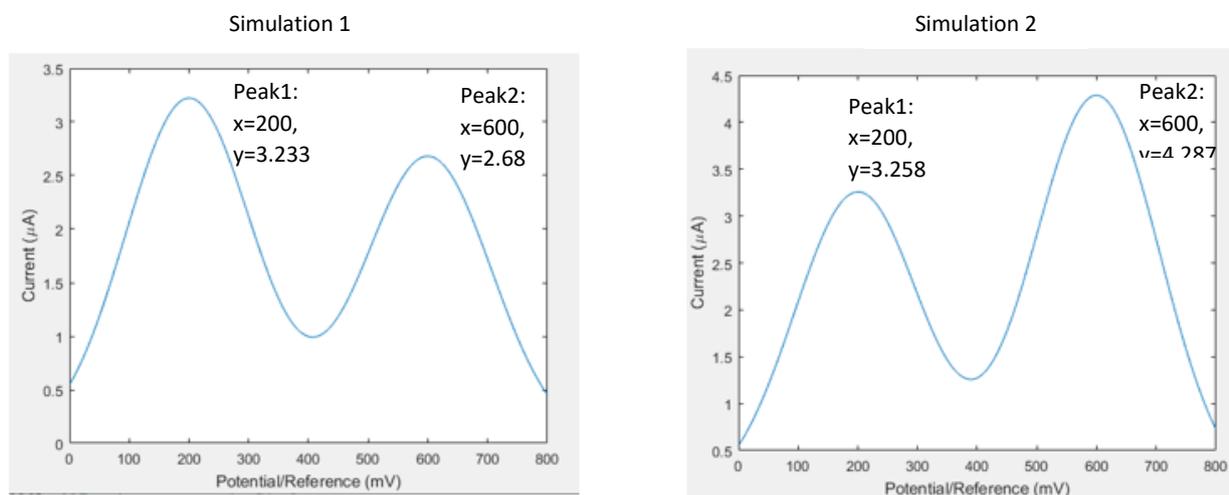


Figure 15. Results of simulations 1 and 2.

The resulting voltammograms are shown in Figure 15. As expected, the current peak 2 is higher in the second simulation, since the related concentration increases. Unfortunately, no significant variations for the Peak1 are recorded.

The test is repeated by maximizing the parameter  $\epsilon$ , simulating the case where the interference between the two drugs is the greatest. Parameters and results are respectively shown in Table 3 and Figure 16.

Table 3

	Simulation 3	Simulation 4
C1 [ $\mu\text{M}$ ]	500	500
C2 [ $\mu\text{M}$ ]	500	800
Position1 [mV]	200	200
Position2 [mV]	600	600
$\epsilon$	1	

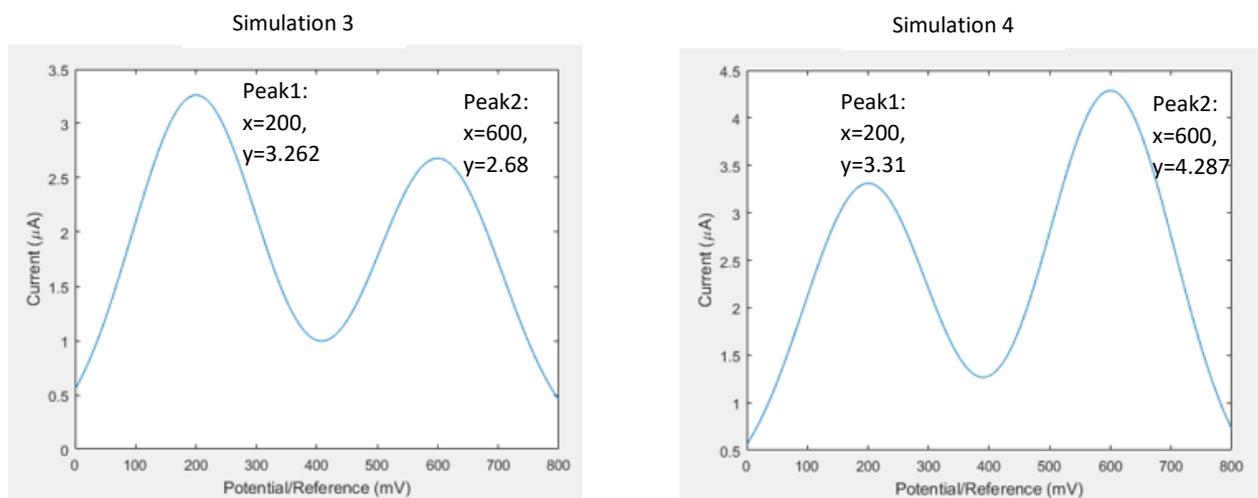


Figure 16. Results of simulations 3 and 4.

Simulations 3 and 4 don't show any improvement. Consequently, tests can be considered failed.

#### 4.1.2 Enzymatic-Inhibition

It is necessary to repeat the same simulations to test the algorithm in case of enzymatic-inhibition. The aim of these tests is to prove a decrease in the current peak of a Drug1 when its own concentration is kept constant whereas the concentration of a Drug2 is increased. Parameters used for simulations 5 and 6 are already shown in Table 2.

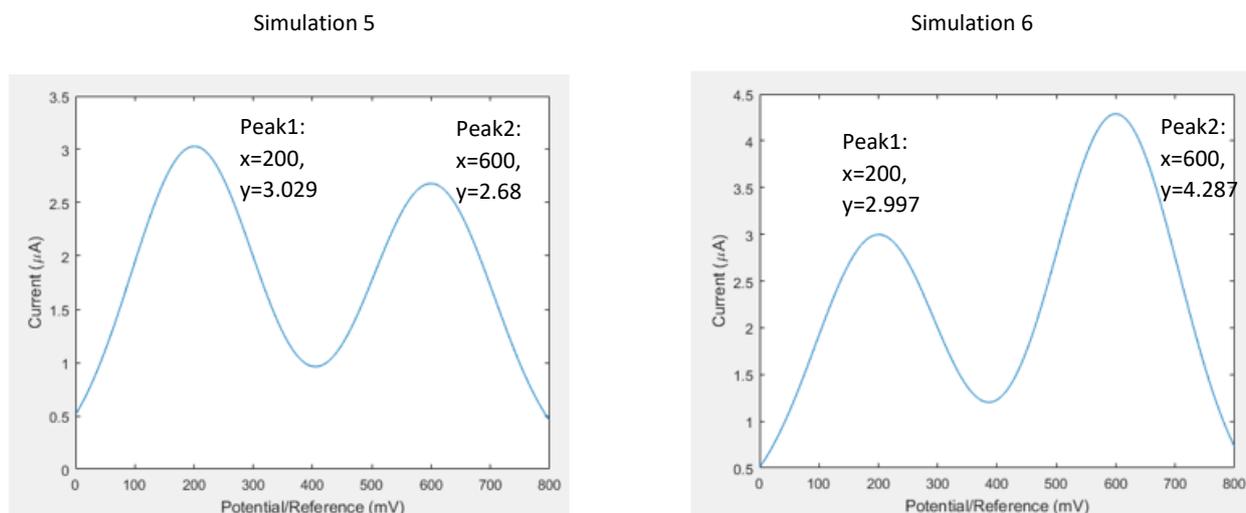


Figure 17. Results of simulations 5 and 6.

In simulations 5 and 6, no decrease in current is recorded for Peak1 when Drug2's concentration increases (Figure 17). Successively, the test is repeated increasing the parameter related to the interference strength as shown in Table 3.

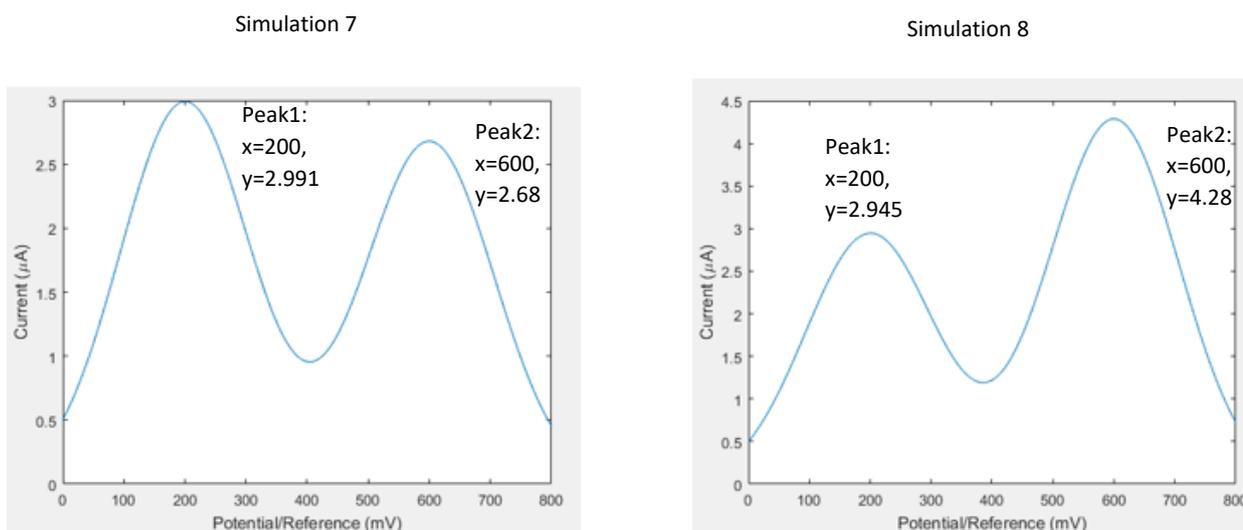


Figure 18. Results of simulations 7 and 8.

Simulations 7 and 8 don't show the expected results. It is concluded that all tests failed.

## 4.2 Corrections

As a consequence of the unsuccessful simulations, a revision of the tool and of the initial theory is required. The code is thoroughly debugged with the aim of finding any mistake. Nevertheless, this procedure doesn't point out significant mistakes. On the contrary, a careful review of the theory published by Carrara *et al.*, with particular focus on dimension analysis of the proposed formulas, highlights an inaccuracy [7]. Indeed, Equation 11 shows

an inconsistency: the interference strength  $\epsilon_j$  is a pure number, ranging from 0 and 1, whereas  $w_j$  is the reaction rate whose dimension is  $\text{nmol}/\text{min} \cdot \text{mg}$  (variation of concentration per unit time). Considering that the result of the equation should be a dimensionless multiplicative factor, an adjustment is necessary.

$$A_k(C_j) = A_{k0} \left(1 - \epsilon_j \frac{w_j}{v_{max}}\right) \quad A_k(C_j) = A_{k0} \left(1 + \epsilon_j \frac{w_j}{v_{max}}\right) \quad (17)$$

Equation 17 is the revised version of Equation 11: The maximum reaction rate  $v_{max}$  is the missing parameter to make the equation consistent. New simulations are carried out to test Equation 17.

## 4.3 Validation

### 4.3.1 Activation

Firstly, a test is carried out to check the right behaviour of “*activation*” function. It is known from literature that Naproxen (NP) activates the catalysis of Flurbiprofen (FL) by cytochrome P450 [7]. Both Naproxen and Flurbiprofen are nonsteroidal anti-inflammatory drugs (NSAIDs). In this test, concentration of Flurbiprofen is kept constant at 200  $\mu\text{M}$ , whereas Naproxen’s concentration increases from 0  $\mu\text{M}$  to 200, 300, 400 and 500  $\mu\text{M}$ . Peaks’ width is artificially decreased to better understand peaks’ variation. In this case, cytochrome P450 peak is not considered.

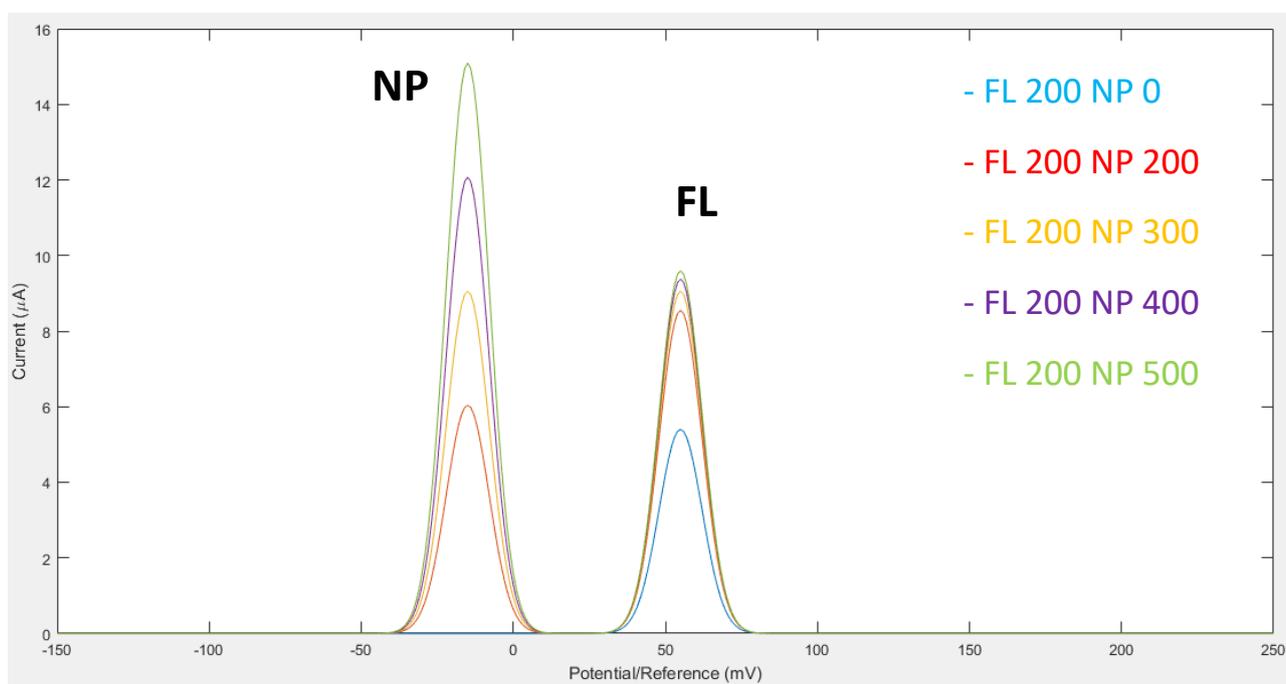


Figure 19. Simulation of Naproxen/Flurbiprofen interaction.

As shown in figure 19, Flurbiprofen's peak amplitude is higher even if its concentration is kept constant. This increase is linked to increasing concentration of Naproxen. It is concluded that this test was successful and "activation" function works properly.

#### 4.3.2 Inhibition

A second test is carried out to try out "inhibition" function. In this case, inhibitory effect of dextromethorphan (DX) on cyclophosphamide (CP) during cytochrome P450-based electrochemical detection is simulated. Dextromethorphan is a cough suppressant whereas cyclophosphamide is a chemotherapeutic agent. In this simulation, cyclophosphamide's concentration is kept constant at 400  $\mu\text{M}$ , whereas dextromethorphan's concentration increases from 0 to 200 and 400  $\mu\text{M}$ . Peaks' width is artificially decreased to better understand peaks' variation. In this case, cytochrome P450 peak is not considered.

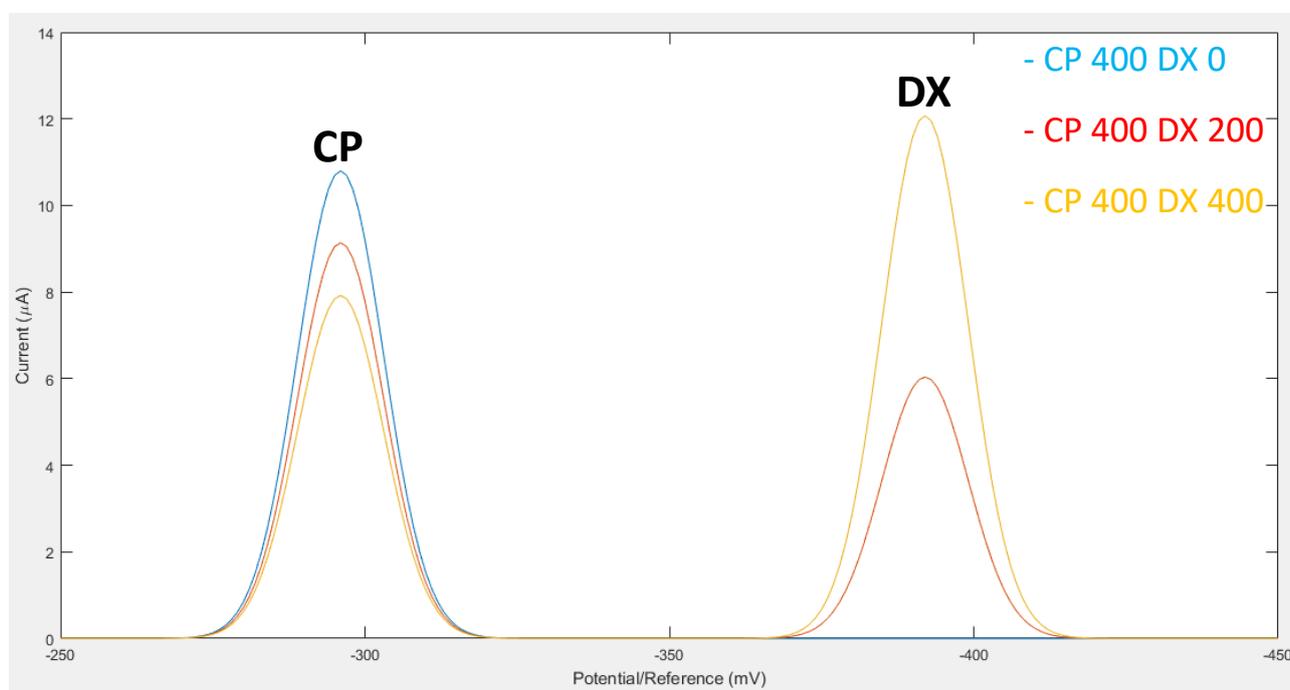


Figure 20. Simulation of dextromethorphan/cyclophosphamide interaction.

In figure 20, it is evident that cyclophosphamide's peak amplitude decreases with the increase of the concentration of dextromethorphan. Thus, test on function "inhibition" is considered successful.

#### 4.3.3 Real cases

Validation of the simulator is carried out by morphologically comparing voltammograms found in literature and simulated ones.

A first comparison is performed for simultaneous detection of cyclophosphamide and dextromethorphan. In this case, real peaks' widths are used to provide a greater likelihood

with reality. Moreover, cythochrome P450's peak is taken into account and set at -380 mV [29]. It is evident from Figure 21 that simulator is capable to represent the same real pattern.

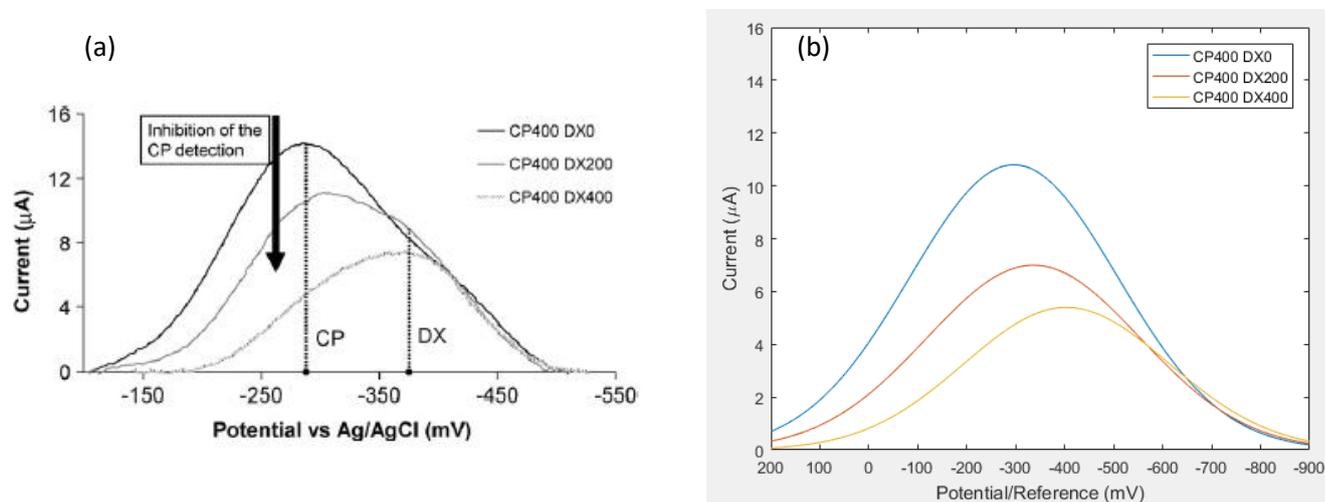


Figure 21. (a) Cyclic voltammogram for simultaneous detection of cyclophosphamide and dextromethorphan. Reprinted from [7]. (b) Simulated CV for cyclophosphamide/dextromethorphan interaction.

Secondarily, a comparison is performed for simultaneous detection of Naproxen and Flurbiprofen. In this case, real peaks' widths are used to provide a greater likelihood with reality. Moreover, cythochrome P450's peak is taken into account and set at -380 mV [29]. It is evident in Figure 22 that simulator is capable to represent the same real pattern.

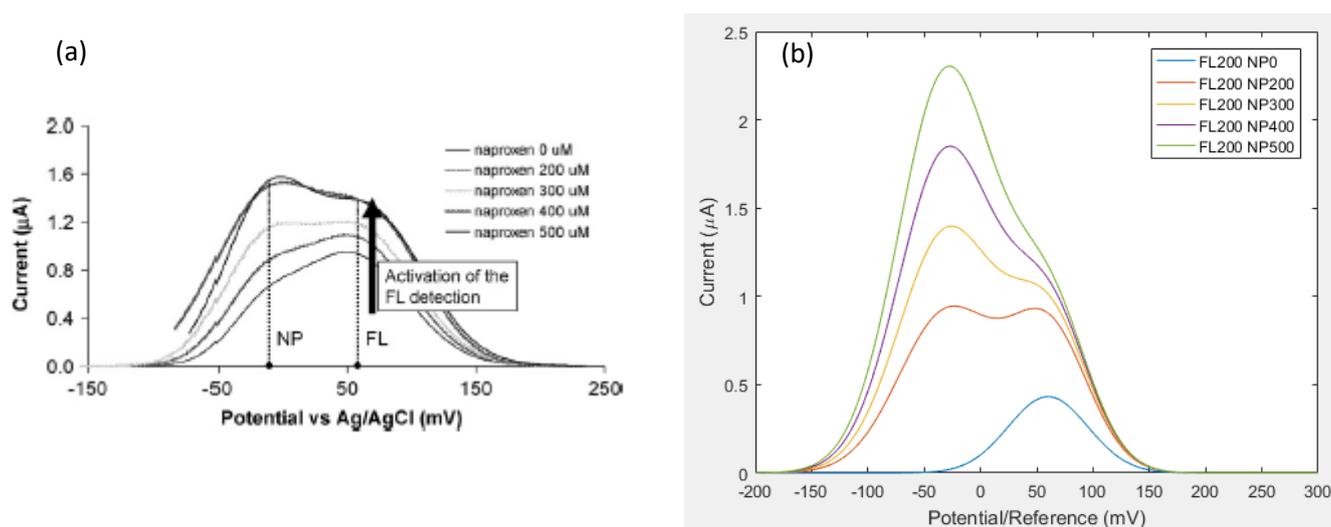


Figure 22. (a) Cyclic voltammogram for simultaneous detection of flurbiprofene and naproxen. Reprinted from [7]. (b) Simulated CV for flurbiprofen/naproxen interaction.

## 4.4 Global sharing

This simulator presents a great potential both for research and educational purposes. In the first case, a scientist will not need to carry out a huge number of experiments in lab anymore: he could search in the database if the simulation he is interested in is already present and use found data to run other simulations. Otherwise, he could add necessary data for simulations and increase information stored in the database. For instance, in order to train a neural network, a great number of examples are necessary to set up a train set. If the aim is to train a neural network to recognize the real concentration of a species from a cyclic voltammogram by excluding the interference, this simulator come to the aid to compensate the lack of experimental data. From educational point of view, students could become familiar with cyclic voltammetry and discover the influence of each parameter on the result, by applying theoretical formula already learned.

Considering all these possible applications, the idea to make the simulator available to the community is born during the development of the tool. Moreover, the freedom to modify the original tool, by adding more functions in the form of modules, could be provided to the user. Python programming language represents the best solution to make it more dynamic and usable as possible. Moreover, a website could be built by including the developed tool.

A first prototype is designed by using Python: a GUI similar to the one designed on MATLAB® is proposed in Figure 23. An extract of main GUI's code developed with Python is reported in Appendix (A.5).

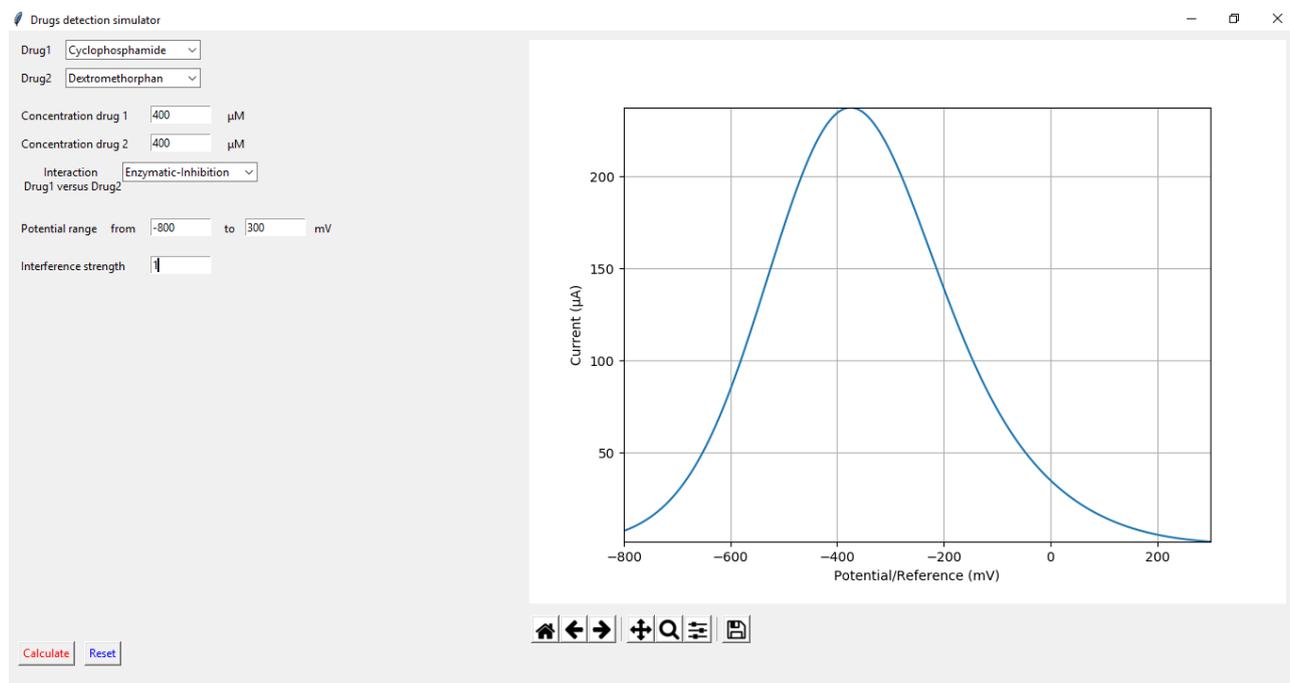


Figure 23. Graphical user interface of the simulator by using Python.

In this Python prototype, a connection with MySQL® database is provided and algorithms to simulate drugs' interaction are included. Future efforts should be addressed to include

additional functions already proposed in the tool developed with MATLAB®. Finally, this tool could be shared worldwide to make full use of its potential.

## 5. Conclusions

The objective of this thesis was the development of a tool aimed to the simulations of drugs detection by means of electrochemical sensors. The reproduced measurements were cyclic voltammograms, where current peaks obtained by scanning potential ranges are linked to the species' concentration in a solution. This behaviour is summed up in Randles-Sevcik equation, which represented the starting point of this research. Gaussian curves were used to approximate Faradaic currents generated by drugs' interactions on cytochromes P450 based sensors.

This work falls within the scope of continuous monitoring of exogenous compounds in oncological patients' blood. Their detection by P450 based sensors shows serious drawbacks consisting in the identification of effective drug's concentrations. The main hurdle consists in interactions between different drugs on the sensor, since cytochromes P450 can be involved in several simultaneous catalytic processes with different substrates. These interactions affect detected currents, in this instance in cyclic voltammetry, and consequently related drugs concentrations.

The proposed simulator could address these difficulties: it will enable the establishment of a dataset made of simulated measurements, useful to train a neural network. The use of artificial intelligence could be the breakthrough to obtain smart sensors for drugs detection. In fact, neural networks trained on simulations could be used to correct data obtained from the sensor and to provide effective concentrations of drugs in patient's blood.

In summary, a simulator and its GUIs were implemented on MATLAB® and subsequently on Python. A database connected to the tool was developed on MySQL® to record drugs and their properties.

Initially, testing on the tool were unsatisfactory: the simulator was not showing expected data and similarity with reality. An accurate revision was carried out to find out the reason, both on the code and on the theory. It was concluded that there was an imperfection in the paper used as literature reference: a discrepancy in dimensional analysis was pointed out and corrected. Successively, the results indicated that simulations had a good likelihood to reality and an effective representation of drugs' interactions.

Future improvements should focus on the enhancement of the database, by incorporating a greater number of drugs and relative features. This study also raises the possibility of creating a website to share the tool worldwide. In this way, scientists around the world could exploit its benefits and help the growth of drugs' database. This tool could be introduced as educational system to better explain electrochemical measurements like cyclic voltammetry and parameters that influence this experiment.

Moreover, further research is needed to take into account other enzymatic kinetics and to implement them. In fact, not all drugs are catalysed by enzymes following a Michaelis-

Menten kinetics. Thus, atypical kinetics and parameters as intrinsic clearances of drugs should be considered.

Finally, I conclude with the prospective that this tool will be exploited in upcoming studies for the development of synthetic datasets to train neural networks. They will be used in future works for the achievement of P450-based electrochemical sensors in exogenous metabolites' detection.

# Appendix

In this appendix, some significant parts of the code developed on MATLAB® and Python are reported.

## A.1 GUI1.m

The *GUI1.m* file contains the code of the main GUI shown in Figure 10. An extract related to the instructions executed when *Calculate* button is pressed is reported.

```
% --- Executes on button press in calculate.
function calculate_Callback(hObject, eventdata, handles)
% hObject      handle to calculate (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
contents_interaction = cellstr(get(handles.popupmenu3, 'String'));
pop_choise_interaction = contents_interaction(get(handles.popupmenu3, 'Value'));
C1 = str2double(get(handles.edit3, 'String'));
C2 = str2double(get(handles.edit4, 'String'));
voltage_start = str2double(get(handles.edit13, 'String'));
voltage_end = str2double(get(handles.edit14, 'String'));
voltage = voltage_start:1:voltage_end;
interferencestrengthinput = str2double(get(handles.edit18, 'String'));
active_area = str2double(get(handles.edit7, 'String'));
%noise
valueno = get(handles.checkbox4, 'value');
valueinput = get(handles.checkbox5, 'value');
valuestandard = get(handles.checkbox6, 'value');
inputnoise = str2double(get(handles.edit16, 'String'));
%variability
valueva = get(handles.checkbox1, 'value');
valuevainput = get(handles.checkbox2, 'value');
valuevastandard = get(handles.checkbox3, 'value');
inputvariability = str2double(get(handles.edit17, 'String'));
[sensitivity, peakposition, peakamplitude, peakwidth, sensitivity2, peakposition2, peakamplitude2, peakwidth2, km2, vmax2] = interaction_data;
[peakposition, peakposition2, peakamplitude, peakamplitude2] =
variability(valueva, valuevastandard, valuevainput, inputvariability, peakposition, peakposition2, peakamplitude, peakamplitude2);
if (strcmp(pop_choise_interaction, 'Enzymatic-Activation'))
    [current] =
activation(C1, C2, voltage, sensitivity, peakposition, peakamplitude, peakwidth, interferencestrengthinput, sensitivity2, peakposition2, peakamplitude2, peakwidth2, km2, vmax2, active_area);
elseif (strcmp(pop_choise_interaction, 'Enzymatic-Inhibition'))
    [current] = inhibition(C1, C2, voltage,
sensitivity, peakposition, peakamplitude, peakwidth, interferencestrengthinput, sensitivity2, peakposition2, peakamplitude2, peakwidth2, km2, vmax2, active_area);
elseif (strcmp(pop_choise_interaction, 'No interference'))
    [current] = interaction(C1, C2, voltage,
sensitivity, peakposition, peakamplitude, peakwidth, sensitivity2, peakposition2, peakamplitude2, peakwidth2, active_area);
end
current = noise(valueno, valueinput, valuestandard, current, inputnoise);
axes(handles.axes1);
plot(voltage, current(:,1));
title('Cyclic voltammogram');
xlabel('Potential/Reference (mV)');
ylabel('Current (\muA)');
```

grid on

## A.2 Readdatabase.m

*Readdatabase.m* file is necessary to connect to MySQL® database. It is automatically executed when the user chooses drugs from database.

```
function [Drugs] = readdatabase
%Set preferences with setdbprefs.
setdbprefs('DataReturnFormat', 'cellarray');
setdbprefs('NullNumberRead', 'NaN');
setdbprefs('NullStringRead', 'null');

%Make connection to database. Note that the password has been omitted.
%Using ODBC driver.
conn = database('MySQL1', 'root', '');

%Read data from database.
curs = exec(conn, ['SELECT  drugs.id'...
    ' , drugs.drugname'...
    ' , drugs.sensitivity'...
    ' , drugs.`peak position`'...
    ' , drugs.`peak amplitude`'...
    ' , drugs.`peak width`'...
    ' , drugs.km'...
    ' , drugs.vmax'...
    ' , drugs.`interference strength`'...
    ' FROM `farmacil`.drugs ']);

curs = fetch(curs);
close(curs);

%Assign data to output variable
Drugs = curs.Data;

%Close database connection.
close(conn);

%Clear variables
clear curs conn
end
```

## A.3 Activation.m

*Activation.m* file contains the instructions to calculate voltammogram's current in case of enzymatic-activation. It is reported below as instance of drugs interaction's evaluation. *Inhibition.m* and *nointeraction.m* files are no reported, but they contain respectively the case of enzymatic-inhibition and the case of no interaction between drugs.

```
function [ITOT] = activation
(C1,C2,V,sensitivity,peakposition,peakamplitude,peakwidth,interferencestrength,s
ensitivity2,peakposition2,peakamplitude2,peakwidth2, km2, vmax2, A)
R = 8.314462618; %%gas constant 8.314462618 in J/mol*K
F = 96485.3365; %%Faraday constant in C/mol
```

```

n =2; %%number of electrons in the electrochemical reaction
v = 20e-3; %%scan-rate in V/s
T = 298; %%Temperature in K (25°C)
%A is electrochemical active area in mm^2
rate= C2/(km2 +C2);
amplitude= peakamplitude*(1+interferencestrength*rate); % + in activation case
I1=zeros(length(V));
I2=zeros(length(V));
ITOT=zeros(length(V));
sensitivity= sensitivity/1000; %sensitivity is provided by user in nA/microM
*mm^2
sensitivity2 = sensitivity2/1000;
for i=1:length(V)
    I1(i)= 0.4463 * sensitivity * A* C1* amplitude* exp(-((V(i)-
peakposition).^2)/(peakwidth.^2));
    I2(i)= 0.4463 * sensitivity2 * A * C2 * peakamplitude2 * exp(-((V(i)-
peakposition2).^2)/(peakwidth2.^2));
    ITOT(i)=I1(i)+I2(i);
end
end

```

#### A.4 Variability.m

*Variability.m* file contains the instruction to take into account random behaviour of electrochemical measurements. *Noise.m* file contains the instruction to evaluate noise effect on current measurements. *Variability.m* function is reported below.

```

function[peakposition,peakposition2,peakamplitude,peakamplitude2]=variability(va
lueva,valuevstandard,valuevainput,inputvariability,peakposition,peakposition2,p
eakamplitude,peakamplitude2)
if valueva == 1 %no variability
    variability = 0;
elseif valuevstandard ==1
    a=(-10/100); % standard variability +-10%
    b=(10/100);
    variability = a + ((b-a).*(rand));
elseif valuevainput==1 %input variability
    a=-(inputvariability/100);
    b=inputvariability/100;
    variability = a + ((b-a).*(rand));
end
peakposition= peakposition *(1+variability);
peakposition2= peakposition2 *(1+variability);
peakamplitude = peakamplitude*(1+variability);
peakamplitude2= peakamplitude2 *(1+variability);
end

```

#### A.5 GUI.py

*Gui.py* file contains the instruction for the main GUI developed on Python. An extract related to related to the instructions executed when *Calculate* button is pressed is reported.

```

def clicked():
    c1 = txt1.get()
    c2 = txt2.get()
    c1 = int(c1)
    c2 = int(c2)
    voltage_start = txt3.get()

```

```

voltage_start = int(voltage_start)
voltage_end = txt4.get()
voltage_end = int(voltage_end)
potentials = np.arange(voltage_start, voltage_end+1, 1)
interferencestrength = txt5.get()
interferencestrength = int(interferencestrength)
A = txt6.get()
A = float(A)
for row in records:
    if row[1] == combo1.get():
        print("Drug is present in the database and it is:", row[1])
        print("Sensitivity = ", row[2])
        print("Peak Position = ", row[3], "\n")
        sensitivity1 = row[2]
        peakposition1 = row[3]
        peakamplitude1 = row[4]
        peakwidth1 = row[5]
        km1 = row[6]
        vmax1 = row[7]
    if row[1] == combo2.get():
        print("Drug is present in the database and it is:", row[1])
        print("Sensitivity = ", row[2])
        print("Peak Position = ", row[3], "\n")
        sensitivity2 = row[2]
        peakposition2 = row[3]
        peakamplitude2 = row[4]
        peakwidth2 = row[5]
        km2 = row[6]
        vmax2 = row[7]

    if combo3.get() == "Enzymatic-Activation":
        ITOT = activation(c1, c2, potentials, sensitivity1, peakposition1,
        peakamplitude1, peakwidth1, sensitivity2, peakposition2, peakamplitude2, peakwidth2,
        km2, vmax2, interferencestrength, A)
    elif combo3.get() == "Enzymatic-Inhibition":
        ITOT = inhibition(c1, c2, potentials, sensitivity1, peakposition1,
        peakamplitude1, peakwidth1, sensitivity2, peakposition2, peakamplitude2, peakwidth2,
        km2, vmax2, interferencestrength, A)
    else:
        ITOT = nointerference(c1, c2, potentials, sensitivity1, peakposition1,
        peakamplitude1, peakwidth1, sensitivity2, peakposition2, peakamplitude2, peakwidth2, A)

a = f.add_subplot(111)
a.plot(potentials, ITOT)
a.axis([voltage_start, voltage_end, min(ITOT), max(ITOT)])
a.set_xlabel('Potential/Reference (mV)')
a.set_ylabel('Current (\u03BCA)')
a.grid()
canvas.draw()

```

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