Universitat Autònoma de Barcelona

Voltammetric sensors and Electronic Tongue for improved sensing of food and pharmaceutical ingredients

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Doctoral Thesis

Doctoral Studies in Chemistry

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Autonomous University of Barcelona

2022

Declaration

Thesis submitted to aspire for the doctoral degree

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Bellaterra (Cerdanyola del Vallès), February 2022

Funding acknowledgement

The present thesis work was carried out in the laboratory of the Group of Sensors & Biosensors of the Department of Chemistry, Universitat Autònoma de Barcelona, with FI fellowship provided by AGAUR, Generalitat de Catalunya and European Social Fund, European Union. Research was financially supported by the Ministry of Economy and Innovation (MINECO) through projects CTQ2016-80170-P, PID2019-107102RB-C21C and the program ICREA Academia.

Grup de Sensors i Biosensors

Unitat de Química Analítica

Departament de Química

Universitat Autònoma de Barcelona

Edifici Cn 08193, Bellatera

ACKNOWLEDGEMENT

This doctoral dissertation would not have been possible without the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this manuscript.

I express my sincere gratitude to my adviser, **Dr. Manel del Valle Zafra**, Professor, Department of Chemistry, Autonomous University of Barcelona for providing me all the guidance and support to undertake my research work. Many thanks for giving me the opportunity to do PhD study under his guidance and for offering professional advice as well as motivation.

I am thankful to **Dr. Xavier Cetó**, Post-doctoral fellow, Sensors and Biosensors Group, Department of Chemistry, Autonomous University of Barcelona for sharing all his experiences and guiding me throughout the study.

I would like to thank all the fellow lab mates **Anna, Andreu, Dioni, Marta, Qing, Mingyue, Noelia and Elena** of Sensors and Biosensors Group, Department of Chemistry, Autonomous University of Barcelona for all the support and help. The affectionate memories with all of them will always be treasured.

I would like to express my gratitude to **Agència de Gestió d'Ajuts Universitaris i de Recerca** (**AGAUR), Generalitat de Catalunya and**

European Social Fund, European Union for providing the FI fellowship. This fellowship has contributed a lot to my career in terms of knowledge gained and skills learnt.

I owe my most sincere gratitude to my family members who are my constant source of support. My **mom´s** love, care and untiring effort to ensure an all-round well-being to myself, cannot be adequately acknowledged in words. This work has seen the light of the day because of her blessings. My husband **Anurag**, his contribution towards this thesis is immense. He stood as a pillar of strength providing me moral support all throughout my stay abroad. I also thank my **father-in-law** and **mother-in-law** for their love and blessings. My sisters **Mitali, Sumi, Ruby, Maina, Rimpi and Runjun** they have been a constant source of support and thanks to their encouragement all throughout the study period. In addition, I thank Runjun for giving me some valuable advice in writing the manuscript.

Last but not the least, a heartfelt thanks goes to all other members of my family, friends and well-wishers, and to the Almighty God, for giving me the strength and showing me the right path.

Munmi Sarma

February, 2022

SUMMARY

SUMMARY IN ENGLISH

This PhD thesis is primarily focused on the improvement of electrochemical sensing properties of graphite epoxy composite (GEC) electrode by means of modification. Graphite epoxy resin composite electrode is the fundamental sensing platform in this electrochemical study i.e. they are the working electrodes in the three electrodes cell configuration. But there are various problems such as noise, fouling, unstable baseline and long response time associated with the sensing of electro-active compounds electrochemically. To solve those problems, it is important to carry out extensive research in search of different modification techniques for working electrodes. Additionally, our final objective in this thesis work was to develop an electronic tongue (ET) with optimal sensors for resolving sample mixtures. For that purpose, essentially we want modified electrodes to obtain differentiated response for the different compounds involved in our electronic tongue applications. This is a necessary condition for electronic tongues: different sensors that provide different signals for the different compounds. Therefore here, in this research, work has been carried out to find some modifiers to be incorporated together with the graphite epoxy composite into the sensor. The effect of this modification on improving the sensing platforms has been studied. Along with modified epoxy resin electrode, possibilities of bare metal electrode in the electrochemical sensing platform have also been studied.

Additionally works has been carried out for a priory selection of optimal sensors for an electronic tongue using canonical variate analysis, principal component analysis and a clustering factor “F”. To illustrate the applicability of the electronic tongue with this a priory selection methodology, simultaneous quantification and discrimination of three different APIs namely Paracetamol, uric acid and ascorbic acid have been demonstrated. The performance of the proposed ET with this a priory selection of sensors was then benchmarked against other previously reported ETs performing the resolution of the same mixtures.

RESUM EN CATALÀ

Aquesta tesi doctoral se centra principalment en la millora de les propietats de detecció electroquímica de l'elèctrode compòsit de grafit epoxi (GEC) mitjançant l'ús de modificadors. L'elèctrode compòsit de resina epoxi i grafit és la plataforma de detecció fonamental en aquest estudi electroquímic, és a dir, són els elèctrodes de treball en la configuració de la cel·la de tres elèctrodes. Però hi ha diversos problemes com ara el soroll, l'enverinament, l'estabilitat de resposta i els temps de resposta elevats associats a la detecció electroquímica de determinats compostos electroactius. Per resoldre aquests problemes, és important dur a terme una recerca de diferents tècniques de modificació i millorar les característiques de resposta dels elèctrodes de treball. A més, el nostre objectiu final en aquest treball de tesi va ser desenvolupar una llengua electrònica (LE) amb una matriu de sensors optimitzada per resoldre mescles de mostres. Amb aquesta finalitat, volem essencialment elèctrodes modificats per obtenir una resposta diferenciada per als diferents compostos implicats en les nostres aplicacions de llengua electrònica. Aquesta és una condició necessària per a les llengües electròniques: diferents sensors que proporcionen senyals diferents per als diferents compostos. Per això aquí, en aquesta investigació, s'ha treballat per trobar alguns modificadors per incorporar juntament amb el compòsit epoxi i grafit al sensor. S'ha estudiat l'efecte d'aquesta modificació en la millora de les plataformes de detecció. Juntament amb l'elèctrode de resina epoxi modificat, també s'han estudiat les possibilitats d'un elèctrode de metall nu a la plataforma de detecció electroquímica.

A més, s'han treballat per a una selecció prèvia de sensors òptims per a una llengua electrònica mitjançant anàlisi canònica de variables, anàlisi de components principals i un factor de clustering "F". Per il·lustrar l'aplicabilitat de la llengua electrònica amb aquesta metodologia de selecció prèvia, s'ha demostrat la quantificació i discriminació simultània de tres ingredients farmacèutics diferents, a saber, paracetamol, àcid úric i àcid ascòrbic. El rendiment de la LE proposada amb aquesta selecció prèvia de sensors es va comparar després amb altres LEs treballades anteriorment que realitzaven la resolució de la mateixa mescla.

RESUMEN EN ESPAÑOLA

Esta tesis doctoral se centra principalmente en la mejora de las propiedades de detección electroquímica del electrodo composite de grafito epoxi (GEC) mediante el uso de modificadores. El electrodo composite de resina epoxi y grafito es la plataforma de detección fundamental en este estudio electroquímico, es decir, son los electrodos de trabajo en la configuración de celda de tres electrodos. Pero existen varios problemas como el ruido, el envenenamiento, la estabilidad de respuesta y los tiempos de respuesta largos asociados con la detección electroquímica de determinados compuestos electroactivos. Para resolver esos problemas, es importante realizar una investigación exhaustiva en busca de diferentes técnicas de modificación y mejorar las características de respuesta de los electrodos de trabajo. Adicionalmente, nuestro objetivo final en este trabajo de tesis fue desarrollar una lengua electrónica (LE) con una matriz de sensores optimizada para resolver mezclas de muestras. Para este propósito, esencialmente queremos electrodos modificados para obtener una respuesta diferenciada para los diferentes compuestos involucrados en nuestras aplicaciones de lengua electrónica. Esta es una condición necesaria para las lenguas electrónicas: diferentes sensores que proporcionen señales diferentes para los diferentes compuestos. Por lo tanto, en esta investigación se ha trabajado para encontrar algunos modificadores que se incorporarán junto con el composite de epoxi y grafito en el sensor. Se ha estudiado el efecto de esta modificación en la mejora de las plataformas de detección. Junto con el electrodo de resina epoxi modificado, también se han estudiado las posibilidades del electrodo de metal desnudo en la plataforma de detección electroquímica.

Adicionalmente se han realizado trabajos para una selección previa de sensores óptimos para una lengua electrónica utilizando análisis canónico de variables, análisis de componentes principales y un factor de agrupamiento “F”. Para ilustrar la aplicabilidad de la lengua electrónica con esta metodología de selección previa, se ha demostrado la cuantificación y discriminación simultáneas de tres ingredientes farmacéuticos diferentes, a saber, paracetamol, ácido úrico y ácido ascórbico. El rendimiento de la LE propuesta con esta selección previa de sensores se comparó con otras LEs trabajadas anteriormente que realizan la resolución de la misma mezcla.

ABBREVIATIONS

Abbreviation	Description of the term
AA	Ascorbic Acid
AI	Artificial Intelligence
ANNs	Artificial Neural Networks
API	Active Pharmaceutical Ingredients
AU	Auxiliary Electrode
CA	Capsaicin
CE	Counter electrode
CPE	Carbon Paste Electrode
CV	Cyclic Voltammetry
CVA	Canonical Variate Analysis
DWT	Discrete Wavelet Transform
EDX	Energy dispersive X-ray
ET	Electronic Tongue

FIA	Flow Injection Analysis
GEC	Graphite Epoxy Composite
LOD	Limit of Detection
PA	Paracetamol
PCA	Principal Component Analysis
PVC	Polyvinyl Chloride
SEM	Scanning Electron Microscope
UA	Uric acid
WE	Working Electrode

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1. INTRODUCTION

1. Introduction

1.1. Sensors

A sensor is a device or subsystem that can detect and/or estimate any changes in its immediate environment and send the data to an electronic device for data processing. Any physico-chemical phenomenon including temperature, light, smell, taste, vibration, humidity, pressure, presence of a chemical substance etc. is transformed to a digital signal by a sensor. The corresponding digital signal is then processed further for displaying, reading or writing depending on the requirement. Chemical sensors are made of three essential components 1. A receptor -to bind the sample 2. An analyte-the target to be detected 3. A transducer- To convert the physical process of recognition to electronic detectable signal. In recent years, sensors have been playing a very widespread role to resolve many aspects in everyday life as there are increasing concerns with issues such as our safety, health, environmental pollution with increasing population [1]. So, there is always in need of developing new technologies that can be incorporated to the field of sensors and can be applicable to our modern life.

1.2. Electrochemical sensor and electrochemical techniques

In an electrochemical sensor, an electrode works as the transducer which send the current produced as a result of redox reaction between a sensing electrode and an analyte to a detectable signal. Among various classes of sensors, electrochemical sensors have been found great interest in several fields of application including food analysis [2][3][4], environmental and industrial monitoring [5][6], medical diagnostics [7], biomedical applications etc. This is because of their numerous advantages such as high sensitivity, accuracy and specificity, fast detection, simple measurement procedure, inherent miniaturization/portability and low cost [8]. Moreover, sample pretreatment is not required in most of the cases, or can be most simplified.

Research has been going on to develop new procedures for analytical measurement techniques based on the principles of chromatography, spectrophotometry etc. to address the challenges of long response times, sample pre-treatment, sample preparation and expensive instrumentation etc. [9]. Recent developments have resulted in new procedures with increased sensitivity and resolution of measurements [10][11]. Ease of handling, portability, and decrease in cost of instrumentation are the other benefits desired from the analytical procedures [12]. For performing on-site analysis of analytes, electrochemical sensors can be a promising tool at a reasonably cheap price with fast and robust results.

Voltammetry is a part of electrochemical methods based on electron transfer reactions [13]. It facilitates the redox reaction of analytes. In this electrochemical technique, the current intensity is measured in a particular range of potential when a polarization is created on the electrical surface [14] The recorded current is obtained from the electron transfers as a result of reaction that takes place between the analytes in solution and the electrode surface in presence of an applied potential. Analytes in solution are either oxidized or reduced depending on the applied potential. Due to the possibility of measuring current intensity over a range of potentials in voltammetric

measurements, without any pre separation steps of analytes, electrochemical study of several analytes in presence of different applied potential can be done simultaneously.

Electrochemical cell is the basic requirement for the electrochemical measurements. It comprises by three types of electrodes: one reference electrode (RE), one counter electrode (CE) (also called auxiliary electrode) and one or more working electrodes (WE) (Figure 1.1).

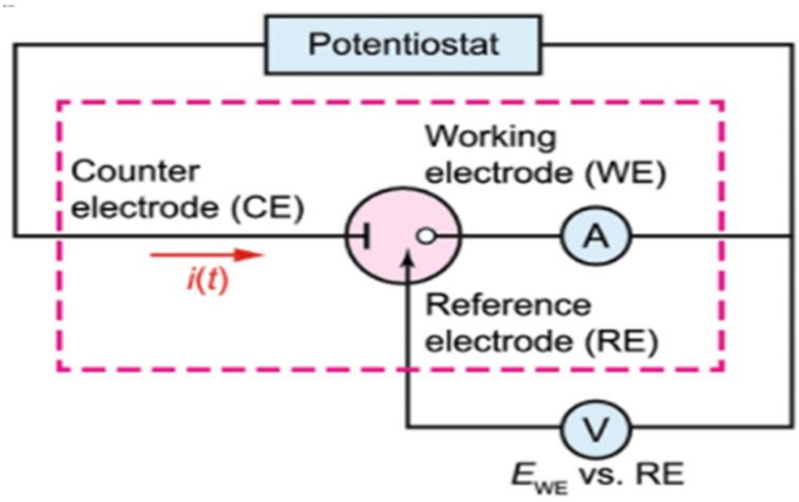

Figure 1.1: Schematic representation of an electrochemical cell arrangement.

These three electrodes cell configuration is used as a source of desired potential during electrochemical measurements as well as measures the current intensity. Working electrode (WE) is the platform where the electrochemical reactions take place and hence the working electrodes should have redox properties on its own. Non polarizable electrode, RE ensures a correct polarization of the electrode and hence is used as a reference with respect to which the potential of other working electrodes can be measured in an electrochemical cell. Counter electrode is used to complete the electrical circuit and current measurements are separated into a CE and RE loop.

1.2.1. Modification of working electrodes

Graphite epoxy resin composite electrode is the fundamental sensing platform in this study i.e. they are the working electrodes in our three electrodes cell configuration. But there are various problems such as noise, fouling, unstable baseline and long response time associated with the sensing of electro-active compounds electrochemically [15][16][17]. To solve those problems, it is important to carry out extensive research in search of different modification techniques for working electrodes. Additionally, our final objective in this thesis work was to develop an electronic tongue with optimal sensors for resolving sample mixtures. For that purpose, essentially we want modified electrodes to obtain differentiated response for the different compounds involved in our electronic tongue applications. This is a necessary condition for electronic tongues: different sensors that provide different signals for the different compounds. Therefore here, in this research, work has been carried out to find some modifying ingredients to be incorporated together with the graphite epoxy composite into the sensor [18]. The effect of this modification on improving the sensing platforms is also studied. In addition of modified epoxy resin electrode, possibilities of bare metal electrode in the electrochemical sensing platform have also been studied. In our research work, actual purpose of finding sensors, modified differently is because modified sensors will be the base of the electronic tongue system [19][20]. For the building of an efficient electronic tongue, various steps need to be considered ranging from selection of proper sensors to selection of chemometric tools. Various modifiers that have been used to improve graphite epoxy composite electrodes in our study are discussed below.

- **Metal oxide nanoparticles:** The materials having at least one dimension in nanoscale are the nanomaterials. Characteristically, they are in between a bulk material and a single atom which makes them to have unique physical, chemical [21] and biological properties [22][23]. Because of their special properties, nanomaterials have been investigated as promising tools in various fields of science and technology including biomedical [24], agricultural [25], solar energy transformation, military technologies, industrial, electronics and many more [26][27]. These applications overall lead to economic and social

improvement of modern life. Nanomaterials differ among them with respect to their different dimensionality. They are classified as zero dimensional (0D), one dimensional (1D) and two dimensional (2D) structures. Nanomaterials with different dimensions exhibit different optical, physical electronic properties because of their quantum confinement effect. A bulk material with quantum confinement along 3 dimensions, results zero dimensional (0D) quantum dots structure where all the 3 dimensions are in nano scale i.e. all of them are within 100 nm. Whereas, quantum confinement along 2 dimension and 1 dimension result quantum wires/nanowires and thin films; respectively. Thus, in 2D nanomaterials, 1 dimension is in nanoscale and other two are in macroscales [28]. Similarly, in 1D nanomaterials only 1 dimension has value of more than 100 nm. Figure 1.2 shows the representation of different nanomaterials with respect to dimensionality.

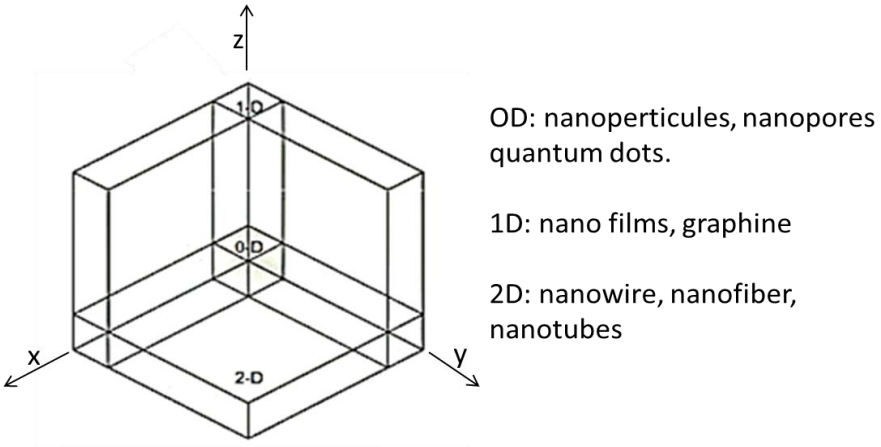

Figure 1.2: Representation of nanometarials with respect to dimensionality.

In this PhD work, among several nanomaterials, some oxide nanoparticles of some metals had been considered for electrochemical application. One of the main reasons of choosing these nanoparticles is because of their encouraging sensory behavior in the analytical field [29] . They have been acquired expansive considerations from a long time and yet there are many possibilities for their applications. These nanoparticles exhibit many special features like chemical stability, controllable size, bio safety, functional biocompatibility etc [30][31]. Moreover, because of their tiny size, they can

enhance the area of the surface of the sensor for the electrochemical reaction to take place. If they are incorporated in the nanopowder form into the electrodes, these metal oxide nanoparticles offer enhance catalytic properties which help to raise the rate of mass as well as electron transfer reaction in the electrode surface. In this way, these metal oxide nanoparticles have potentials to strengthen the analytical performance of the electrodes in terms of selectivity and specificity.

Metal oxide nanoparticles that were taken into account for the purpose of electrochemical sensing application using electronic tongue in this PhD work are oxide nanoparticles of titanium (TiO_2), bismuth (Bi_2O_3), tin (SnO_2) and zinc (ZnO).

- **Redox catalysts:** Redox catalysts have been playing a vital role as electrocatalysts for electrochemical study of many electroactive compounds. Therefore two redox catalysts: Prussian blue and cobalt(II) phthalocyanine were considered for modification of our graphite epoxy composite electrodes. Electrochemical properties as well as their applications are discussed below.

  1. **Prussian blue:** Prussian blue $Fe_4[Fe(CN)_6]_3$) has been used extensively in the field of electroanalytical chemistry for improvement of various sensors such as glassy carbon, graphite, carbon nanotubes with respect to their analytical parameters [32][33][34]. Modification of such kind of electrodes with Prussian blue, also makes the electrochemical measurements safe from the disturbance by the interfering agents. Because of their good electrocatalytic activity, ability to transfer electron reversibly and excellent redox activity they have been proved to be of great use for determining chemical and biological analytes such as proteins [35], glucose [36], nitrites [37] ref and many more. It is widely recognized as ‘artificial peroxidase’, because of its efficiency as a transducer for electrochemical oxidation reduction reaction of hydrogen peroxide (H_2O_2) [38]. Applications of Prussian blue in sensors field come from their zeolytic character. It exists as a porous coordination polymer and forms a face-centered cubic lattice where Fe^{2+} and Fe^{3+} are

present in the lattice points. Fe^{2+} and Fe^{3+} are bridged by cyanide groups (linear) where N of nitrogen atoms from the cyanide group octahedrally coordinated to the Fe^{3+} ions whereas Fe^{2+} ions are surrounded by the carbon from the cyanide groups (Figure 1.3). These arrangements result an open framework structure where there are 25% vacancies of Fe^{2+} ions in the unit cells [39][40]. These vacancies allow intercatalation of small molecules or ions when Prussian blue is reduced during electrochemical measurements.

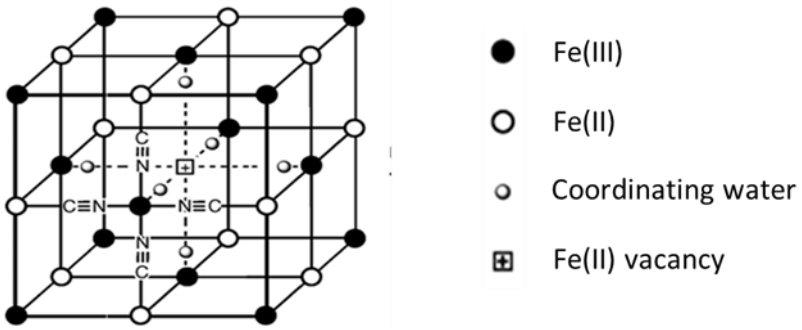

Figure 1.3: Prussian blue in face centered cubic arrangement.

2. **Cobalt(II) phthalocyanine:** Phthalolocyanines are belonging to the class of 16 membered (8 nitrogen, 8 carbon) macrocyclic compounds. These aromatic organic compounds have a conjugated Π system of 18 electrons and four subunits of isoindole, which are connected in a cyclic order by nitrogen atoms in meso positions (Figure 1.4). The ring is flexible enough and several functional materials including metal ions can be fitted into the central cavity Metallophthalocyanines formed in this way have been used as a sensing platform due to their high thermal stability, significant absorbance in ultra-violet regions due to $\Pi \rightarrow \Pi^*$ transitions, redox activity, photovoltaic effect etc. These properties make them applicable in various areas such as in thermal printing screens, as material for semiconductors, photovoltaic cells and many more [41]. In addition to those, due to their enhance catalytic properties, electrochemical sensor modified with phthalocyanines have been found

to be effective in the analysis carried out by methods such as potentiometric, amperometric, voltammetric and flow injection analysis (FIA) for detecting electroactive analytes in very low level [42]. Among the metalloporphyrines, various working electrodes such as glass electrode, carbon paste electrode (CPE), glassy carbon electrode (GCE), multi walled carbon nanotube paste electrode (MWCNTPE) have been found to be effectively modified with metallophthalocyanines having transition metals such as Co(II), Ni(II), Cu(II), Fe(II) and Fe(III) etc [43]. These metallophthalocyanine modified electrodes could detect many analylytes like phenolic compounds, heavy metals, primary acids, or drug substances at very low concentration level.

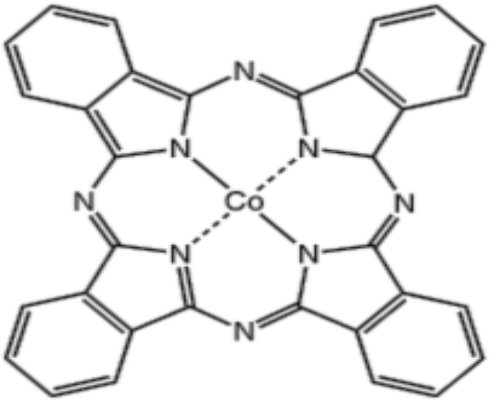

Figure 1.4: Structure of Co(II) phthalocyanine.

Additionally due to their chemical stability in a wide potential range as well as in a wide pH range, those metallophthalocyanine modified electrodes can resolve complex mixture by electrochemical measurements. Their catalytic efficiency proves to lower the peak potential and increases peak intensity of a particular electroactive compound during electrochemical measurements making the entire electrochemical methods rapid and cost effective [44]. Here in this research Co (II) phthalocyanine was taken for modification of graphite epoxy resin electrode for development of an electronic tongue.

- **Polypyrrole:** Polypyrrole is belonging to the group of conducting polymers. Optical and electrical properties of conducting polymers are similar to sometimes metals or to inorganic semiconductors depending on physicochemical properties. Accordingly, they have been found to have the potential to be used in sensing platform for fabricating various electrochemical sensors [45]. Among the conducting polymers, the one which have been extensively used in electroanalytical field is polypyrrole due to its low toxicity, higher surface area, cost effectiveness etc. In addition to those advantages, polypyrrole being the most important among the conducting polymers to be used in sensors field is because of its monomeric unit which is pyrrole [46]. The presence of aromatic pyrrole rings connected to one another, the polymer creates conjugated system and the electrons are delocalized in the polymer. These conjugations lead to inherent electronic properties such as tunable, environmentally friendly, flexibility, easy synthesis procedure etc [47]. Because of these properties, modification of conventional electrodes with polypyrrole brings to better analytical performance in them with respect to change of electrical resistivity/conductivity during electrochemical measurements of electroactive analytes [48]. Unlike many of other modifiers, application of polypyrrole in the sensors field is not only limited to the study of the samples in the liquid state but various gas sensors for detecting toxic gases such as ammonia (NH_3) [49], hydrogen sulphide (H_2S) [50] and many other volatile organic compounds [51] etc. has been established using polypyrrole as the sensing material. Figure 1.5 shows the structure of polypyrrole.

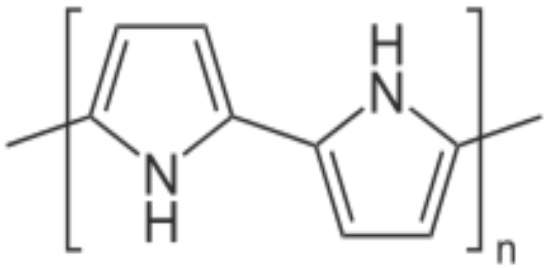

Figure 1.5: Structure of polypyrrole.

1.3. Electronic tongue (ET) approach

An electronic tongue can be defined as the combination of an array of various sensors having low selectivity and up to date mathematical approach for data processing [20]. These data processing is based on Multivariate data analysis and/or Pattern Recognition – Principal Component Analysis (PCA), Artificial Neural Networks (ANNs), etc. [52]. Hence this analytical system comprises of

- An array of sensors having cross-response properties and/or having low selectivity that gives manifold data with added analytical information.
- Advanced chemometric processing tools based on multivariate data analysis and/or Pattern Recognition for extraction and interpretation of the relevant features from the multiplex readings.

Increasing demand of informatics in daily life encourages developing new tools and features, based on advanced computational software. Analytical chemistry is always in need resolving maximum dataset in minimum time possible [53]. In addition, there is always in need of techniques which can work in every circumstance irrespective of problems related to specificity and selectivity. Concept of electronic tongues is improving now a days to resolve complex mixtures of analytes [54].

Analytical systems have widespread application in different fields such agriculture, food, medicine and industries, requiring highly efficient devices capable of resolving complex mixtures. Natural world always inspires to develop biomimetic set up based on combining mathematical treatments and physical sensors. A branch of analytical chemistry thrives to design systems that are inspired or taking clues from three mammal senses namely sight, taste and smell, thereby developing efficient analytical tools. Therefore, devices such as electronic eyes, electronic noses and electronic tongues are being developed having wide application [55]. Electronic tongue, as the name suggests is an analytical system or device whose principle and design are inspired from a biological tongue, a highly specialized sense organ of vertebrates which perceives the signal of taste. A human tongue was initially thought to have specialized zones which perceives different tastes, like sweet taste is perceived by the tip of the tongue, sour and salty on the sides and bitter on the back [56] (Figure 1.6). However, in

reality it is proven that the whole of tongue can sense different tastes, while the most sensitive part of the tongue is the tip, edges and back of the tongue. An exception being the back of the tongue, which is more sensitive to bitter taste, which might give functional utility to perceive bitter, spoiled or poisonous food or materials and giving final alarm signals to the brain before its intake.

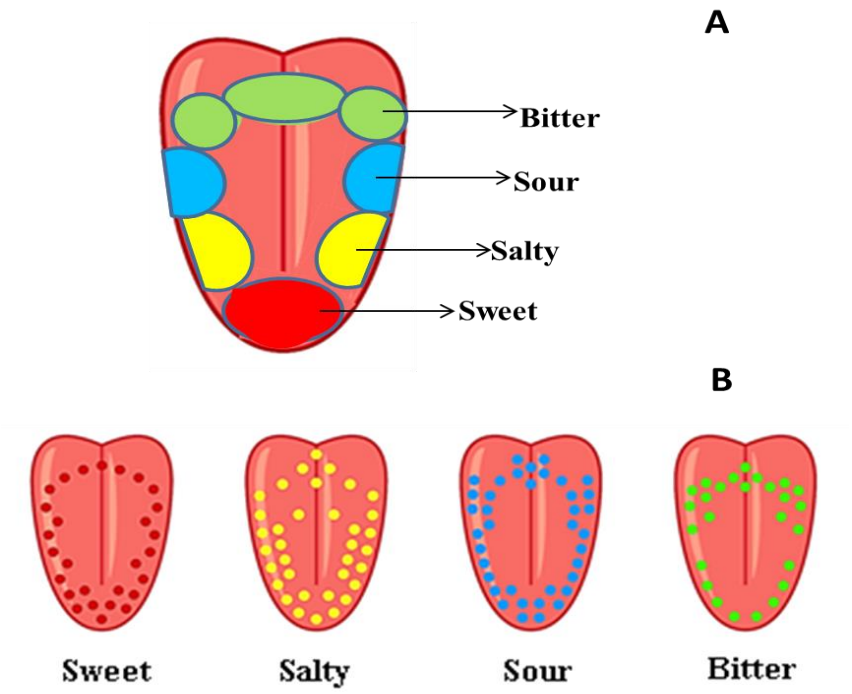

Figure 1.6: (A) Traditional representation of specialized zones in a tongue which perceive different tastes. (B) Actual manner of taste perception by a tongue, where the whole of tongue can perceive different tastes.

Since, the whole of the tongue can sense different taste signals, visualizing the tongue as a sensor or device, the tongue is ideally composed of an array of non-specific and low selective sensors, which is essentially the concept deployed in electronic tongues in sensor assembly. In fact, electronic tongues thrive to mimic, replicate or refine the functional mechanism of evolutionary complex taste sense organ and signal processing of brain present in vertebrates, to develop highly sophisticated analytical tools. Hence, there is high degree of functional resemblance between a biological tongue and an electronic tongue assembly. However, in case of a biological tongue the signal of taste is perceived by the taste buds which are collections of nerve-like cells that connect to nerves running into the brain and the brain processes the input based on

memory or experience over the years. In contrast, the role of nerve cells is performed by sensor array in electronic tongues, which collects the signals and depending on the complexity of the sample, can generate small to large amount of data, interpreted by various mathematical procedures to understand the collected data. A scheme showing functional resemblance between biological tongue and electronic tongue is shown in Figure 1.7.

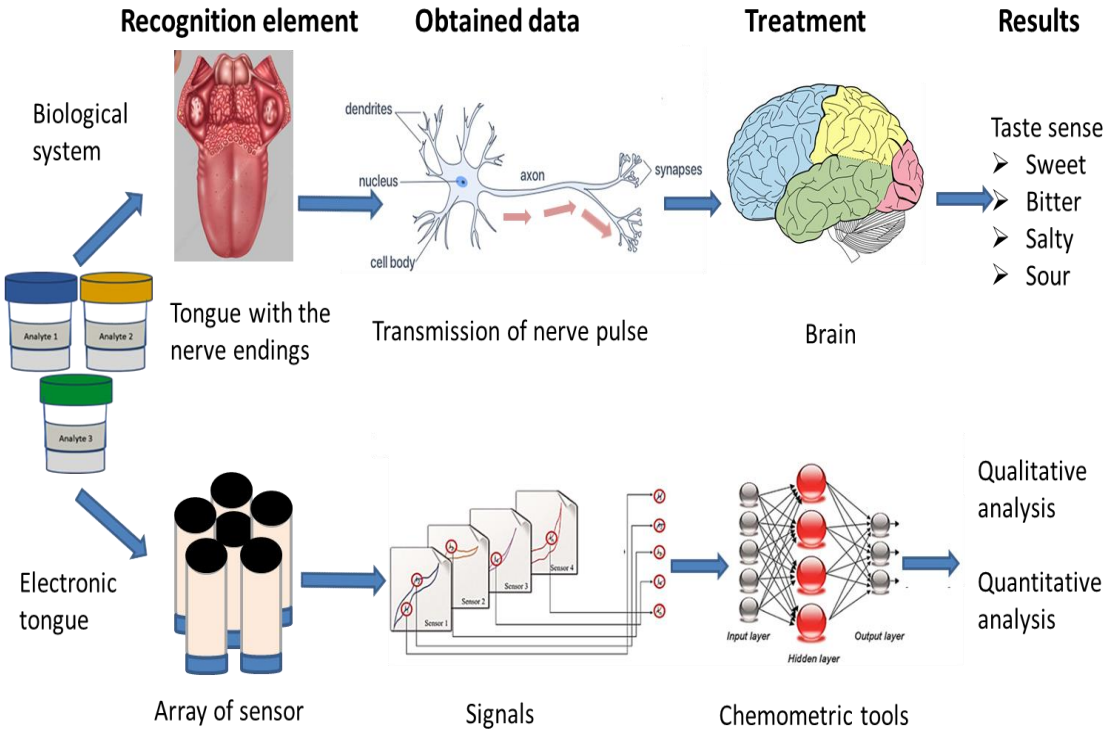

Figure 1.7: Functional analogy of electronic tongue with biological tongue.

After treading the signal with mathematical procedure, Vlasov first proposed the idea of electronic tongue in late 90s' to describe an array of sensors. Different chemometric methods such as partial least square, principal component analysis, artificial neural network, canonical variate analysis are developing day by day to resolve complex dataset obtained from analytical measurements [57]. In the present PhD work, wherever needed, voltammetric sensors form the array of sensor for building the electronic tongue system.

Although the idea of electronic tongue is associated with non-specific and non-selective sensors previously, but the current research is going to include some aspects of specificity and selectivity to the sensor array according to their detection abilities of the analytes. This leads to the improvement of the electronic tongue for discriminating complex solutions containing analytes of different physicochemical properties. This improvement encourages the development of sensors of different types by incorporating various elements such as metal oxide nanoparticles, crown ethers, molecular imprinted polymers, enzymes or many types of elements.

1.3.1. Use of electrode array in ET

Sensors are the building blocks for the electronic tongue system. They are the units of the electronic tongue where the electrochemical reaction takes place and they convert external stimulus into a human readable digital signal. Since, analytical field of research mostly deals with quantitative study, so sensors should be capable of producing quantifiable signal from chemical, physical or biological information of the substances under study. Hence, one should be very much careful while selecting a sensor for an electronic tongue.

Sensors used in the electronic tongue system should always have some characteristics such as high resolution, low response time, low signal to noise ratio (S/N), high sensitivity and selectivity, reversibility and absence of hysteresis etc. Proper study of a certain analyte mainly depends on the performance of the sensor having these characteristics. In addition to those parameters, one should also consider other factors including portability, low cost, easy fabrication method, long lifetime, wide linearity range, repeatability, reproducibility and so on depending on the final objective of analytical work.

It is not always possible to get the best sensor characterized by all the necessary qualities as mentioned above. An alternative way needs to be proposed to overcome such situations. Every sensor is not capable of sensing well different types of samples in every measuring environment. Hence, one needs to design specific sensor which can respond to a particular analyte in the mixture in a controlled way, irrespective of other

interfering analytes present in the sample. But it is not always possible to design such kind of sensors in a straightforward methodology. Very often, additional procedures such as pre-treatment steps, where we can mask or eliminate certain interfering agents, become necessary [19]. However, this may be time and resource consuming and also may need trained professional which reduces the overall efficiency of the analytical procedure.

To overcome those limitations, a new trend of using a sensor array, instead of using a single sensor has shown promising results [58]. Since each sensor can have different response towards the sample matrix, they can give a large set of information to be treated. At the same time, we can bypass many problems explained above, that arises while using single sensor. Hence, in this way partially specific or sometimes even unspecific sensors can be merged together to get information of a sample matrix which may contain components of different properties. Data processing tools and signal treatment field are developing day-by-day to resolve the set of information from the multivariate calibration model. In this manner, electronic tongue systems which uses sensor array in combination with chemometric tools becomes promising methodology to overcome many problems that arises during study of complex sample matrix [59](Figure 1.8).

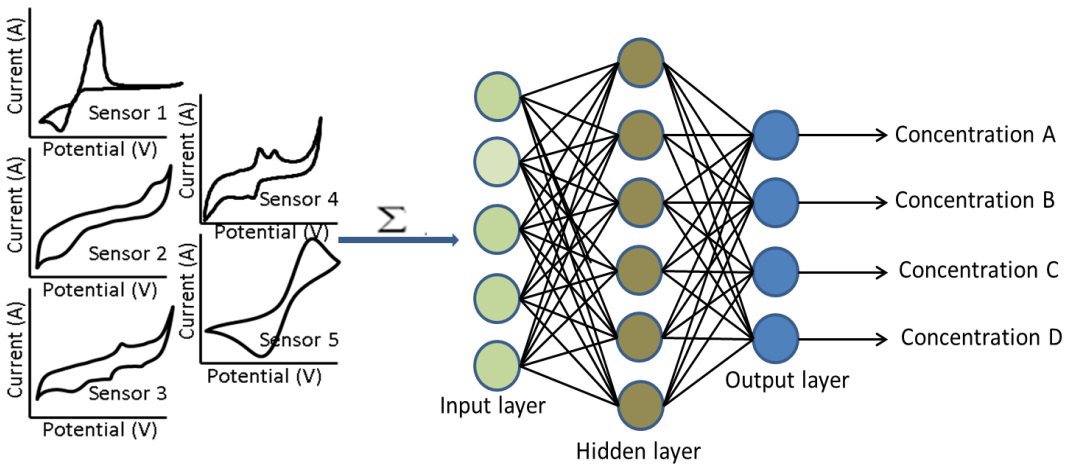

Figure 1.8: Use of sensor arrays in electronic tongue system.

Depending on the types of sensors used in the array of an electronic tongue, for resolving a particular sample matrix, sensors can be classified as follows [19].

- **Sensor array of specific sensor**s: This kind of sensor array has some specificity, i.e., the array contains sensors, each of them are selective to different analytes of the sample matrix. This helps in simultaneous determination of various components in the mixture.
- **Sensor array of redundant sensors**: Here, sensor array is formed with same kind of sensors and hence increases the efficiency and robustness in the method.
- **Sensor array of unspecific sensors**: Here, sensor array is formed with unspecific sensors with low selectivity that present a cross-response with differential sensitivity towards a certain number of analytes. This allows the simultaneous determination of the chemical species present in a complex sample with the use of processing tools to correct the interferences that occurs between them. Therefore, this strategy gives us a powerful tool to extract information of our system, which in addition allows us to obtain information that is related to a perception like the smell or the taste senses.

1.3.2. Chemometric analysis

In the recent years, there has been a growing interest for data analysis, based on artificial intelligence (AI). Machine learning, chemometrics and deep learning are promising AI based methods that resolve high dimensional experimental data into simpler ones which can be linked to specific analysis [60]. According to international chemometric society definition “chemometrics is a chemistry discipline that uses mathematical and statistical methods to design or select tailor-made methods and experiments, to provide maximum chemical information by means of data analysis” It is a branch of study, which is mainly associated with the computational analysis [61]. There is increasing demands of chemometrics analysis now-a-days because of capabilities of processing complex dataset into simpler ones. Along with many other fields, chemometric tools are of great use in the field of analytical chemistry as well as in normal works with large scale analytical purposes. For example, analysis of x samples with an array of x sensors using voltammetric technique gives data with high

dimensionality and complexity. To resolve such kind of large-scale data chemometrics is found to be the best solution [62][63]. The principles of the chemometric parts that are used in this work are explained in sections given below.

1.3.3. Data compression in voltammetric electronic tongue

As mentioned above the original dataset of a particular experiment for example having x sensors and x measurements is very large. Building a quantitative model for resolving the complex sample matrix using such large dataset and with this high dimensionality is not possible. Therefore, before processing, the original large-scale dataset needs to be compressed to smaller ones. There are certain chemometric tools that can compress the dataset without losing information from the original signal [64]. Here, in this PhD work the analytical technique that was used to carry out the measurements is cyclic voltammetry. Hence, wherever needed, the compression of the large scale cyclic voltammetric data was performed with the tool namely, Discrete Wavelet Transform (DWT) [65]. Before compressing the data with DWT, cyclic voltammetric signals were unfolded as shown in the Figure 1.9.

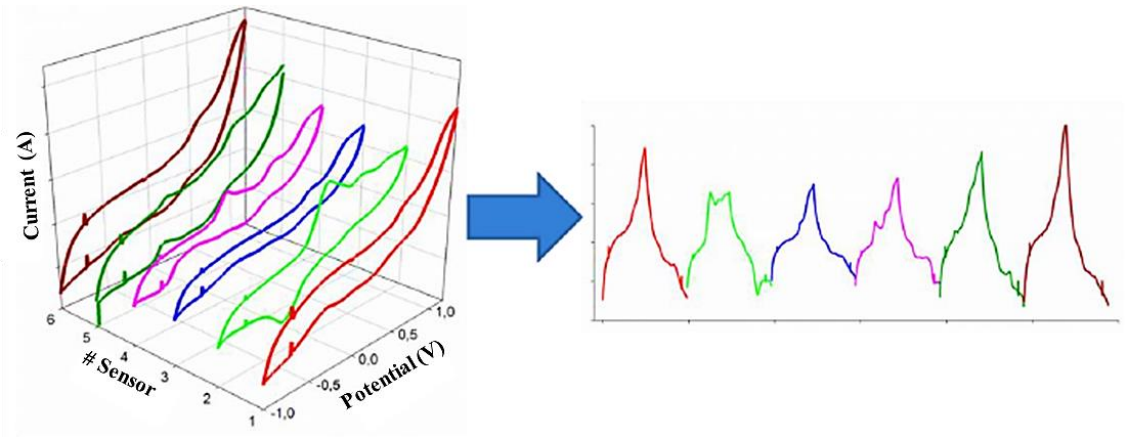

Figure 1.9: Unfolding of cyclic voltammograms

1.3.4 Principal component analysis

Principal component analysis (PCA) is a qualitative chemometric tool that provides visual representation of a complex dataset. It uses mathematical rearrangement of the complex dataset and tries to maximize the sample variability. As human eye cannot visualize a dataset having higher dimension, PCA is the tool to reduce the dimensionality and makes visualization of dataset easy [66]. As shown in the figure 1.10 PCA helps grouping of the similar datasets from a mixed complex dataset of high dimensionality [67].

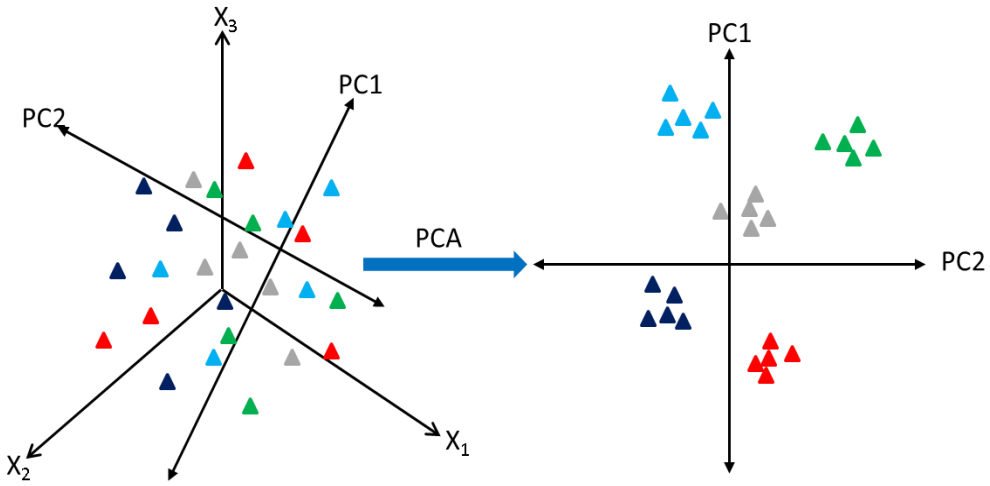

Figure 1.10: Schematic representation of data transformation through PCA.

The main idea behind the PCA can be simply understood by taking the example of cells from breast growths. Those cells are either benign or cancerous. Now if we measure the parameters from the images of the cells, we will need to measure a brunch of parameters from the images such as how big the cells are, how bumpy they look, how big their surfaces, how big is their radius, how round they are etc. Let's say there are 30 parameters like that. But to plot each parameter between cancerous and benign are not practically possible. Even though we plot for 30 parameters, but we will not have a proper idea of distinction between the two types of cells from so many plots. In addition to this, there can be situation in another dataset which may have 100 or 1000 of parameters. In such cases PCA found to be the solution which reduce those 30 or

1000 dimensions into 2. Now we can make 1 plot with this 2 dimensional data and overall we will be able to have an idea from the 30 or 1000 of dataset and can visually distinguish the benign and malignant cells. Figure 1.11 is the PCA plot obtained from "Breast Cancer Wisconsin dataset" and we can see there are some differences between the two types of cells as both the types of cells are found to be in cluster in the PCA instead of mixture of the points together.

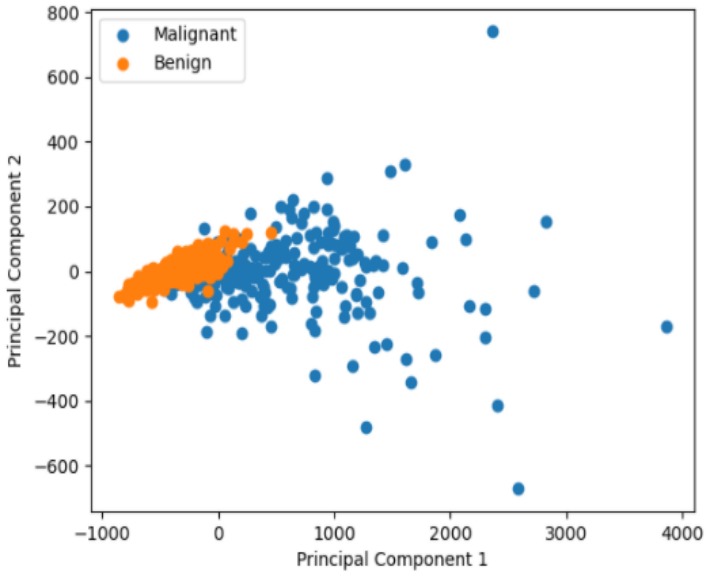

Figure 1.11: PCA score plot obtained from the cells found in breast growths, 1 dot represents 1 cell.

In brief, overall steps regarding the mechanism of PCA are

- Normalization of the original large scale data points.
- Covariance matrix X calculation from the data points
- Calculation of eigenvalues and eigen vectors.
- Arranging the eigen vectors with respect to their eigen values in decreasing order.
- Choosing of new k dimensions from 1st k eigen vectors.

We can see visually how PCA works using the above steps by taking the example of famous iris flower set. Dataset consists of 4 parameters: petal length, petal width, sepal length, sepal width from three different kinds of irises Versicolour,

Virginica and Setosa. Before performing PCA analysis into the dataset three classes represented by blue red and green points are like mixing all together and it's not possible to visually distinguish them. But after performing PCA analysis it we could see 3 clusters representing 3 different kinds of irises. Moreover, as we can see in the figure 1.12, first 2 principal components that are PC1 and PC2 are enough to describe the dataset. So if necessary we can remove the PC3, PC4… and so on.

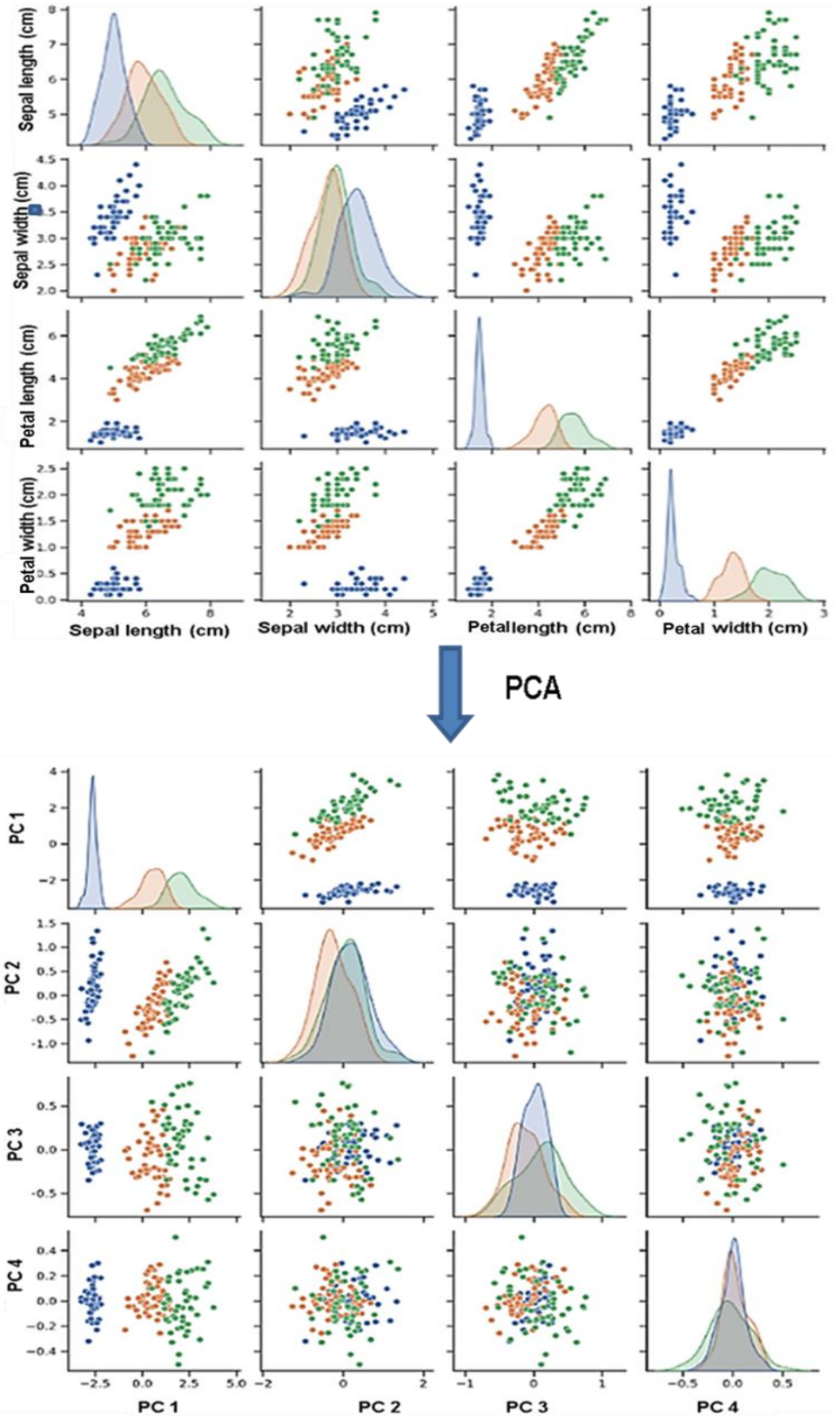

Figure 1.12: Transformation of Iris dataset with principal component analysis.

1.3.5. Artificial Neural Networks

To facilitate efficient processing of data generated by a sensor array of electronic tongues from complex samples, Artificial Neural Networks (ANN) come into play. These ANNs are bio-inspired mathematical/statistical models having the capability of processing large amount of data, bearing close resemblance to data processing by biological networks such as the mammal brain. The establishment of ANNs to the chemometric field, resolves various multivariate sample matrix with respect to qualitative and quantitative purposes. This provides errorless experimental results to a great extent. If we correlate mammal's brain with this chemometric tool we can see the similarity with neuron and perceptron. As neurons are the basic unit of the nervous system for transmitting and receiving signals from different parts of the body, perceptron is the independent logic unit for building the ANN model. The perceptron, acting as decision making unit in ANN model, consists of more than one input (x_n) connections and a single output (a_k). Each of the input units is associated with a determined weigh (W_i) and whenever needed a bias (b_k) is applied [68] [69]. The perceptron gets activated when the sum of the weighted inputs attains a definite threshold $T_{ki,}$ and produces an output signal. Schematic representation of resemblance between a neuron and a perceptron is shown in the figure 1.13.

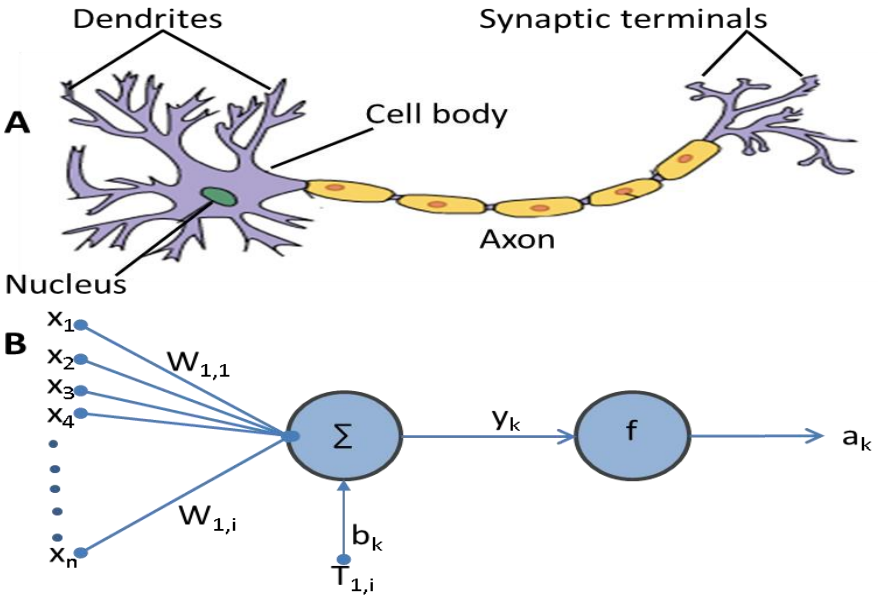

Figure 1.13: Schematic representation of neuron (A) and perceptron (B).

Moreover, perceptrons exist as different layers in ANNs, and each layer is consisting of perceptions that share the same inputs. In this way, multilayered ANNs can be formed. In general, multilayered ANN is buildup of mainly three layers input layer, hidden layer and output layer connected in a same direction [70]. The input layer is directly connected to the dataset obtained from the original experiment. Without any modification of data, input layer transforms the information to the next layer i.e. the hidden layer. Hidden layer processes the data obtained from the input layer and transforms the data to the output layer. The output layer provides the information of the analytes used in the experiment [71]. For example, if the original dataset obtained from an experiment associated for resolving a sample matrix containing three analytes, the output layer will provide three results corresponding to the three analytes.

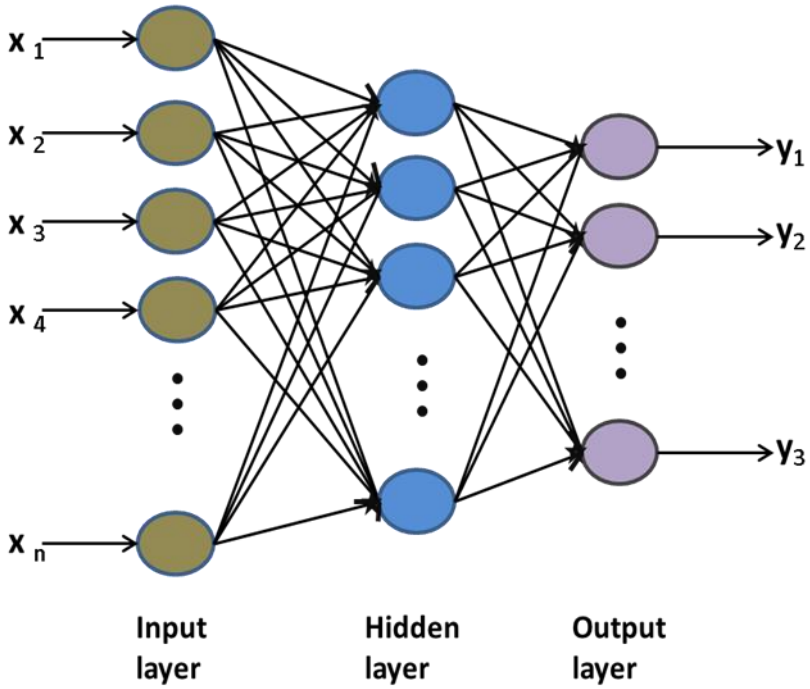

Figure 1.14: Scheme of multilayered ANN.

If we see the working scheme of ANN, we can say that basically ANN is alearning model. Training or learning step is the key stage for building an ANN model. Therefore, to use ANN for a complex data set, first we need to train the model with some known inputs, then it is able to process unknown inputs of same kind. For example, in case of resolving mixture of analytes, first we need to make the training set by mixing the analytes in different known concentration to train the model. This is followed by random choosing of some standard samples to test the ANN model.

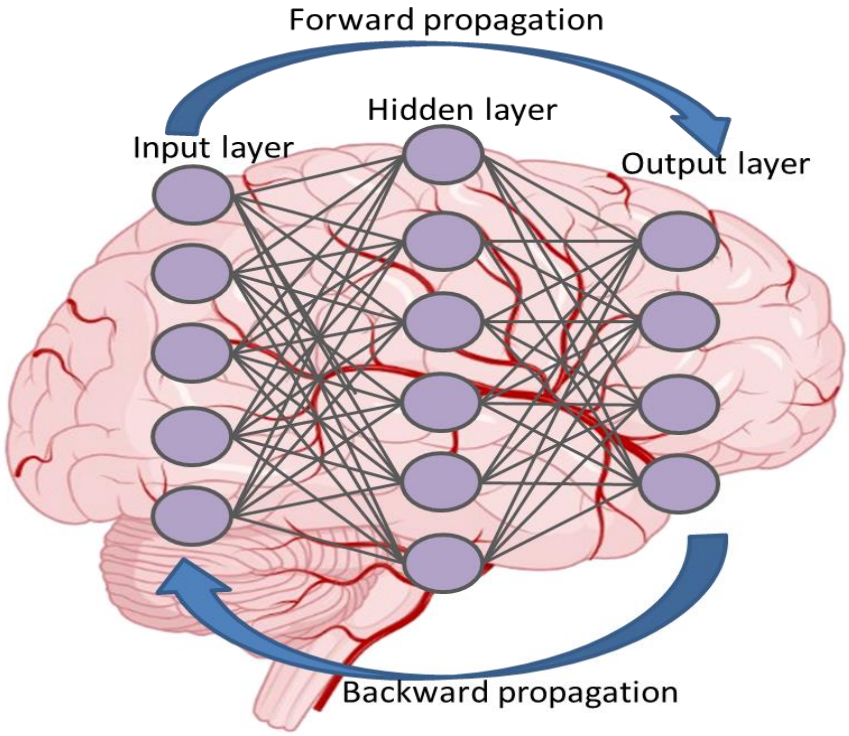

Figure 1.15: Scheme of a ANN model designed for mixture of three analytes with " back-propagation algorithm" which consist in a first forward propagation on inputs and then back propagation of the errors allowing the inputs to be re-weighted and start the loop again until the predicted values and known values become closest, allowing in this way the learning process.

To verify if the system is working properly or not the result obtained from the output layer needs to be studied. ANN model is considered to be trained properly if the experimental results from the testing samples are as expected. In case of error, a term "back propagation algorithm" comes to play [72]. In backpropagation algorithm, the error needs to be considered and propagated in the backward direction to adjust the prediction. Backpropagation involves the changing of numeric weight given for each data, and so on until it reaches to the minimum error possible [73][74]. In the next step,

we can process the data obtained from the mixture containing the samples of unknown concentrations. Since the ANN model had been trained and tested previously with the known concentrations of the analytes, the model will be able to resolve the mixture with unknown concentration of the analytes.

To check the applicability of our developed graphite epoxy resin composite electrodes modified with different modifiers explained above in the section 1.2.1, two study cases had been taken. These study cases were chosen by taking into account of analytes which are used in large scale in our daily life.

The first study of this thesis deals on electrochemical estimation of the phenolic alkaloid capsaicin, which is found in chili peppers. Chili peppers are belonging to a horticultural commodity for adding pungency or hot, spicy flavour to the dishes [53]. Capsaicinoids are naturally occurring lipophilic alkaloids present in chili peppers and their presence is the main reasons of hot or spiciness in chili peppers. Capsaicin and dihydrocapsaicin are the two major capsaicinoids present in most varieties of hot peppers [75]. Chemically, capsaicin is trans-8-methyl-N-vanillyl-6-nonenamide and it is an odourless, off white, fat soluble, solid having molecular weight of 305.4 $g \cdot mol^{-1}$ and melting point of 62–65°C [76].

In addition to its usage in preparations of food, capsaicin has also been used in the industry of pharmaceuticals because of its medicinal properties [77]. It is a well-known anti-oxidant and found to have anti-carcinogenic, anti-bacterial, anti-mutagenic, anti-tumoral properties [78]. Capsaicin is also used as an additive in drugs for controlling cholesterol, obesity and in topical formulations used for pain relief [79]. Additionally, for self-defense, skin conditioning, and for controlling mob violence by security authorities, pepper sprays based on capsaicin have been widely used [80]. Its first isolation was in 1919 and from then researches are going on for extraction, estimation and applicability of this highly valued alkaloid.

As the 2^{nd} study case in this research work, there was an investigation of the use of canonical variate analysis (CVA) and principal component analysis, in combination with the F factor clustering metric, for the a priori tailored selection of the optimal sensor array for a given electronic tongue application. The former (PCA, CVA) allows

us to visually compare the performance of the different sensors, while the latter (F factor) allows us to numerically assess the impact that the inclusion/removal of the different sensors does have in the discrimination ability of the electronic tongue (ET) towards the compounds of interest.

The reason of taking into account of the priori selection of sensors in the electronic tongue is because, despite of its importance, most of the papers dealing with ETs focus on the developed application itself or the data treatment stage, but very few reports on the choice of the sensors. The challenges here arise on the a priori selection of the best combination of sensors that can carry out the desired qualitative or quantitative task given the difficulty to assess the cross-reactivity shown between them and the impact that this will have in the final model [52]. Therefore, the development of a simple methodology that allows the a priori selection of the optimal sensor array to carry out a specific application is of utmost interest.

In order to demonstrate and illustrate such a procedure, the simultaneous quantification of paracetamol, ascorbic acid, and uric acid mixtures were chosen as the study case. These mixtures correspond to a common case in the pharmaceutical field where the determination of paracetamol in the presence of ascorbic acid was attempted. The latter is usually present as an excipient, whereas the inclusion of uric acid is motivated as some studies suggest that ascorbic acid intake is related to uric acid concentration in serum. This particular case was chosen as this mixture has already been previously analysed in the Sensors and Biosensors Group laboratories, with employ of different sensors arrays, thus providing us with guidance on which performance could be expected and whether previous results could be improved or not by tailoring the electrode choice to particular cases.

In this direction, study was carried out to demonstrate the advantages derived from the tailored selection of the sensor array for each ET developed application. Upon measurement of stock solutions of the different active pharmaceutical ingredients (APIs), those were transformed by using PCA/CVA, which in combination with the F factor metric allowed the selection of the different sensors. Next, a quantitative model was built by means of artificial neural networks to achieve the simultaneous

determination of the three APIs, the performance of which was benchmarked against previously reported ETs.

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2. OBJECTIVES

2 Objectives

Objectives that were considered for this doctoral thesis are

1. To modify graphite epoxy composite electrodes with different modifiers for improvement in their electrochemical sensing characteristics as well to get differentiated responses for different analytes.

2. To study the applicability of modified sensors with respect to improved electrochemical sensing of certain electroactive analytes namely capsaicin, paracetamol, uric acid and ascorbic acid.

3. To gain preliminary ideas on application of chemometric tools such as ANNs and PCAs that has been used in our research group.

4. To evaluate methods for a priori selection of optimal sensors to be used in an electronic tongue sensor array.

5. To develop an electronic tongue with optimal sensors for simultaneous quantification of ascorbic acid, paracetamol and uric acid in mixtures.

3. EXPERIMENTAL

3. Experimental

3.1. Materials

All the chemicals and commercially available analytes were used without any additional refinement. Capsaicins as well as two of the API's namely uric acid and paracetamol were acquired from Sigma Aldrich (St. Louis, MO 63118, USA). Ascorbic acid was acquired from Panreac Química SLU (Barcelona, Spain). For preparation of graphite epoxy composite electrodes, graphite powder with particle size of less than 50 µm was obtained from BDH Laboratory Supplies, United Kingdom. The corresponding hardener and the Epotek resin H77 were purchased from Epoxy Technologies, Billerica, USA. All the chemicals required in the preparation of glycine buffer (pH 2.5) and phosphate buffer (pH 7, were acquired from Merck, Darmstadt, Germany. Various modifiers, such as cobalt (II) phthalocyanine, polypyrrole and oxide nanoparticles of tin, zinc, bismuth and titanium that have been used to improve the sensing performance of the graphite epoxy composite electrodes were obtained from Sigma Aldrich, St. Louis, MO 63118, USA. Whereas Prussian blue was obtained from Acros Organics, Geel, Belgium. Ultrapure water purified with a MilliQ (Millipore, Billerica, MA, USA) system was used for the sample solution preparation as well as preparation of buffers. Real samples which were taken in the 1st study case of this PhD work are- a pharmaceutical cream and two commercially obtainable sauce samples. The pharmaceutical cream used is Alacapsin, which is an analgesic cream manufactured by the pharmaceutical company Alfasigma (Spain). The sauce samples are Tabasco Hebanero Hot pepper from the company, Mcllhenny (Avery Island, USA); and Delhuerto hot pepper from Peru.

3.2. Apparatus and chemometric software

A Crison micro pH 2002 pH-meter from Crison instruments, Barcelona, Spain was used for adjustments of pH in the entire work. A PGSTAT 30 Autolab 6-channel potentiostat (EcoChemie,The Netherlands) was used for accomplishing all the electrochemical measurements. This Autolab potentiostat works with software of GPES 4.7 version (EcoChemie). An electrochemical cell with an arrangement of three electrodes (reference, auxiliary and working electrodes) was used for the analytical measurements. Here, reference and auxiliary electrodes are associated in a single body combined electrode. An Ag/AgCl reference electrode and a wire of metallic platinum constitutes the combined electrode the platinum wire acts as the auxiliary electrode.

Chemometric analysis were carried out with the use of Matlab 7.1 (MathWorks, MA, USA) and Statistics Toolbox and Neural Network Toolbox built in packages. OriginPro 2017 and Sigmaplot were used for drawing the regression lines as well as the analysis of results.

3.3. Working electrodes construction scheme:

As explained in the section 1.2.1 of introduction part of the thesis, modified graphite epoxy composite electrodes were used for the working electrode purposes. The word composite in the working electrodes is associated with the composite material [1][2]. Composite materials are made by mixing two ingredients having different properties. Properties of overall composite materials are different from the properties of each of the ingredient material. Here, in this study composite material is corresponding to the mixture of epoxy resin and graphite particles where graphite particles are distributed in a matrix formed by epoxy resin. The resultant composite material is a strong conductive material [3]. Construction scheme of each of the modified electrodes are summarized in the following steps-

- A copper disc with a diameter of 5 mm was cut and dipped into a solution of diluted HCL for cleaning. After cleaning and drying the disc, an electrical connector was joined into the copper disc by soldering.

- The copper disc in combination with the electrical connector is then inserted into a polyvinyl chloride (PVC) tube of having internal diameter of 6 mm. This insertion leads to the formation of a cavity of cylindrical shape. The depth of the cavity is around 4 mm which would be filled by a paste of composite material along with a specific modifier
- The paste was prepared by taking 2% of specific modifier, 15% of graphite powder along with the epoxy resin and the corresponding hardener in the ratio of 20:3. These additives were then mixed by hand gradually for 40-45 minutes to get a homogenous paste. The paste is then inserted into the previously fabricated cylindrical cavity like a cake like shape as shown in the figure 3.1(B) and then the resulting unit was kept under the temperature of 80°C in oven for 3 days.
- In the final step, surface of the electrode was polished to get a shiny flat surface. The polishing was performed with the use of emery papers with decreasing grain size.

One of the main advantages of such kind of composite electrode is that, if there are any loss of electrochemical responses in the electrode surface during measurements, the surface of the electrode can be regenerated by re-polishing the corresponding surface. Figure 3.1 shows the construction scheme of modified graphite epoxy composite electrode.

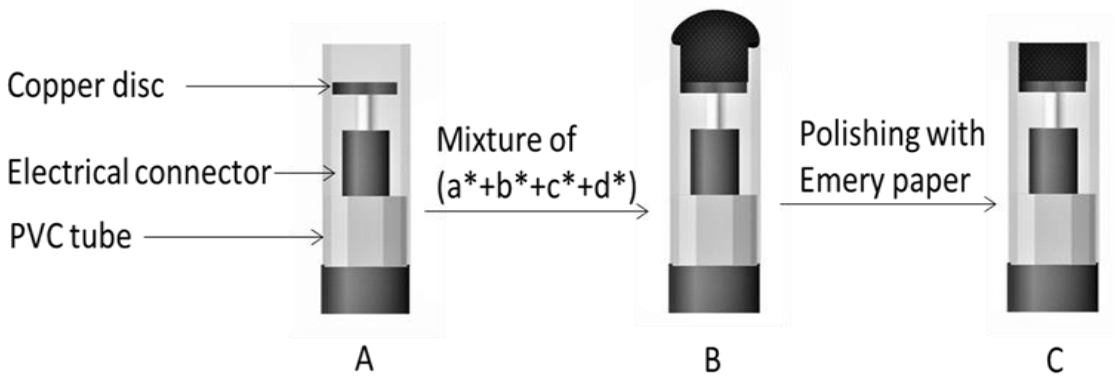

Figure 3.1: Modified graphite-epoxy composite electrode construction scheme. a: graphite powder, b: specific modifier, c: Epoxy resin d: Hardener.

Using the above procedure as shown in figure 3.1, seven differently modified graphite epoxy composite electrodes were prepared for investigating their applicability in the electrochemical analysis. Modifiers used for constructing different electrodes are

- Titanium((IV) oxide (TiO_2) nanoparticles,
- Bismuth (III) oxide (Bi_2O_3) nanoparticles,
- Zinc (II) oxide (ZnO) nanoparticles,
- Tin (IV) oxide (SnO_2) nanoparticles,
- Prussian blue (PB),
- Cobalt (II) phthalocyanine (CoPc) and
- Polypyrrole (PPy).

In addition to above 7 modified GEC sensors, a metallic electrode of platinum had also been investigated for the purpose of electrochemical sensing application. This metallic electrode was prepared by taking a small wire of platinum (99.95% pure) with a diameter of 1 mm. An electrical connector is connected to one end of the wire by soldering method. The soldered unit is then inserted into a PVC tube (dimeter 6 mm). The empty part of the tube is then filled with epoxy resin in order to fit the wire inside the PVC tube in such a way that only cross-section of the wire is being exposed for electrochemical sensing [4][5].

Electrochemical applicability of these kinds of electrodes (modified GECs and Pt electrode) was investigated by taking into account of two study cases. Results of these two study cases were published in two articles as mentioned above. Hence experimental parts associated with both of these study cases are explained separately in the following sections.

3.4. Study case 1: Determination of capsaicin using a TiO_2 nanoparticles modified electrode.

This work was to verify the applicability of a modified composite electrode for improved sensing of the lipophilic alkaloid capsaicin (CA). Research was carried out to find a better modifier for the improvement of the sensing performance of composite

electrode in capsaicin determination. Nanoparticles of titanium dioxide (TiO_2) were found to be effective in this study. Experimental steps involved in this work of electrochemical sensing of capsaicin are described below.

3.4.1. Preparation of samples and buffer

Glycine buffer of strength 0.2 $mol \cdot l^{-1}$ with KCl (0.1 $mol \cdot l^{-1}$) as saline background was chosen to be used in the entire electrochemical study of capsaicin. Glycine buffer was chosen as an outcome of pH study where optimum pH for electrochemical capsaicin determination TiO_2 modified sensor was evaluated. As capsaicin (CA) is not wholly soluble in water, mother solution of capsaicin was made in ethanol-water solution having 48% ethanol. Sample solutions for electrochemical measurements of capsaicin were prepared by diluting the mother solution of CA in glycine buffer. For determination of optimum pH, Britton Robinson buffer having KCl (0.1 $mol \cdot l^{-1}$) as saline background was used.[6]

3.4.2. Extraction of capsaicin from the real samples.

Real samples used to check the applicability of TiO_2 modified sensor towards the real sample analysis are described in section 3.1. As Capsaicin is not wholly soluble in water, extraction from real samples is carried out using 96% ethanol. Extraction process was initiated by taking 2.5 g of each of the real samples in a beaker. 35 mL of ethanol (96%) is then added to the beaker and the solution is then allowed to sonicate for 15 minutes. After sonication, solution is then stirred for 2 hour using a magnetic stirrer[7][8]. Afterwards, the solution containing the extracted capsaicin was filtered through a glass-fiber filter paper (GMFA SCHARLAU Ø 90 mm). The filtrate was collected in a volumetric flask of 50 ml. In the next step, the volumetric flask containing the capsaicin extracted filtrate was filled up to the mark with solution obtained from washing the funnel having the solid part from the real sample[9]. This rinsing step was performed to extract all the capsaicin that may present in the solid part of the real sample in the filter paper. Extracted solution obtained in this way is then used for electrochemical measurements.

3.4.3. Voltammetric measurements

Voltammetric measurements were done with the cyclic voltammetry technique, between the potential window of -0.9 V and +1 V vs. Ag/AgCl. Step potential and scan rate values considered for the measurements were 0.01 V and 100 $mV \cdot s^{-1}$; respectively. Each measurement was followed by a cleaning step where electrode surface was stirred for 60 seconds after being dipped in a specific media of ethanol-water solution having 48% ethanol. Sample solutions of CA in different concentration were prepared by diluting mother solution of CA using glycine buffer having 24% ethanol.

For determining optimum pH to be taken for the entire study, 40 µM CA solutions were prepared separately in different pH (2 to 7) in Britton Robinson buffer and measurements were carried out according to the above experimental procedure.

To check the behavior of TiO_2 modified sensor towards the electrochemical sensing of capsaicin in glycine buffer, sample solution of CA in a concentration of 60 µM was prepared by diluting mother solution of CA by glycine buffer having 24% ethanol. Here, Glycine buffer was used as a result of pH study. Electrochemical measurements were performed using the procedure as explained above.

Repeatability and stability study of the electrode towards capsaicin determination were also carried out. For repeatability study, 10 consecutive measurements of capsaicin in carried out in a repeated manner. Stability study was performed by measuring blank buffer and 35 µM capsaicin solutions in a cyclic manner (14 cycles). Between each cycle of measurements, the surface of the electrode was cleaned using a media of ethanol-water solution (48% ethanol) by stirring for 1 minute.

For measuring the extracted real samples, first of all extracted samples were diluted using 0.266 $mol \cdot l^{-1}$ glycine buffer up to 4 times. The dilution was performed to get the same background electrolyte solution (0.2 $mol \cdot l^{-1}$ glycine with 24% ethanol) as the previous electrochemical experiments in this study. Electrochemical measurements were then performed by taking the extracted samples followed by the standard additions of CA in order to evaluate the exact amount of CA in the real samples [10].

3.5. Study case 2: Determination of a paracetamol, ascorbic acid and uric acid with a voltammetric electronic tongue.

In this experimental work, eight different kinds of electrodes were first considered. Using canonical variate analysis (CVA), principal component analysis (PCA) and a clustering factor "F", a number of optimum sensors out of 8 sensors were selected for the formation of an electronic tongue system. Sensors that were initially considered for investigating their applicability to form an ET are 7 modified graphite epoxy composite sensors/electrodes (GEC electrodes) and a metallic electrode of platinum. Each GEC electrode was separately modified with, Prussian blue, Polypyrrole, Cobalt (II) phthalocyanine and oxide nanoparticles of titanium (TiO_2), bismuth (Bi_2O_3), zinc (ZnO) and tin (SnO_2). After choosing the optimum sensors for the ET, it was then verified for resolving sample matrix containing 3 active pharmaceutical ingredients (APIs) including ascorbic acid, paracetamol and uric acid in their different concentrations. Mechanistic steps involving to carry out the entire study are summarized below.

3.5.1. Preparation of Samples and electrochemical Measurements

Phosphate buffer (KH_2PO_4+K_2HPO_4) in the strength of 0.05 M with KCl 0.1 M as saline background was chosen to be used in the entire electrochemical study. Sample solutions of all the APIs were prepared by diluting their mother solution using phosphate buffer. Voltammetric responses of all the 8 sensors towards API solutions, each of having strength of 250 μM were separately evaluated to find out the best sensors to be used in the ET. The electrochemical characterizations of the selected optimal sensors were carried out by measuring API sample solutions separately. In this way, the analytical parameters namely- sensitivity, linearity, reproducibility, detection limit (LOD), etc. were evaluated for each selected sensor with respect to all the three APIs.

For resolving all the three APIs from their sample matrix a set of electrochemical measurements were carried out. Sample solutions prepared for this

simultaneous analysis were prepared in two subsets. The subsets are training subset and the testing subsets represented with blue and yellow circles respectively in the figure 3.2. The former was used for building a quantitative model [11][12][13], while the later one was for assessing the real performance of the built model. The quantitative model was constituted on the basis of a 3^3 factorial (tilted) design with 27 samples. On the other hand, there were 11 samples in the testing subset and were randomly oriented along the investigational area (figure 3.2).

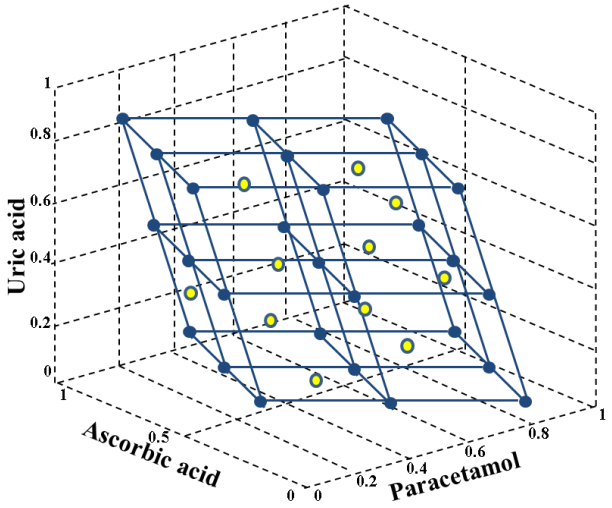

Figure 3.2: Schematic representation of 3^3 factorial design including training (blue circles) and testing (yellow circles) subsets.

Voltammetric measurements were done following the cyclic voltammetry technique between the potential window −0.7 V and +1.2 V vs Ag/AgCl. Step potential and scan rate value considered for the measurements were 10 mV and 100 $mV \cdot s^{-1}$; respectively. The measurements were carried without considering any accumulation time as well as pre-conditioning step. Additionally, any drifts or fouling effects that may arise throughout the measurements were minimized by carrying out a measurement in the blank solution of phosphate buffer after every sample measurement.

All the voltametric measurements were carried out using the above analytical situations except the experiment for selecting the optimal sensors. In the process of selecting the sensors, the potential from −1.5 V to +1.5 V (with step potential:10 mV,

scan rate: 100 $mV \cdot s^{-1}$) was considered. The reason of taking this wide potential range was to find out any probable voltametric peak.

3.5.2. Chemometric Analysis

Recorded voltammograms were first compressed with the help of discrete wavelet transform (DWT), which allowed to decrease the dimensionality of the data while preserving the relevant information [14]. This is followed by CVA and PCA. Both the techniques were used for qualitative analysis as well as for selection of optimal sensor array. At last, ANNs were built for simultaneous determination of API from samples containing the API of different concentrations.

CVA, PCA analysis and calculation of a clustering factor F was carried out to find out the optimal sensors to be used in the ET. PCA and CVA analysis was used for the visual mapping of the sensing performance of all the 8 sensors towards the API. whereas, the calculation of F factor was used to numerically measure how addition or elimination of the different electrodes affects the discrimination capability of the electronic tongue.

F factor is the ratio of variances among various clusters and the summation of internal variance in all the clusters [15][16].

$$F = \frac{\frac{\sum_{i=1}^{k} n_i (\overline{z_i} - \overline{\overline{z}})^2}{k-1}}{\frac{\sum_{i=1}^{k} \sum_{j=1}^{n_i} (z_{ji} - \overline{z_i})^2}{\sum_{i=1}^{k} n_i - k}}$$

Where k is the number of classes, i the number of following class, j the following number of the sample in i-th class, n_i the number of samples in i-th class, and z_{ji} the sensor response for j-th sample in i-th class, and $\overline{z_i}$ and $\overline{\overline{z}}$ are the mean value of a sensor response in a particular class of samples and the mean value of sensor response for all samples, respectively. These can be defined as:

$$\overline{z_i} = \frac{\sum_{j=1}^{n_i} z_{ji}}{n_i}$$

$$\overline{\overline{z}} = \frac{\sum_{i=1}^{k} \sum_{j=1}^{n_i} z_{ji}}{\sum_{i=1}^{k} n_i}$$

Calculation of F factor may be applied as a standard method for the measurement ability of a definite sensor to differentiate among various set of samples considered in electronic nose and electronic tongue [ET] applications [17].with increasing F values, the discrimination between different classes becomes easier.

After the selection of optimal sensors using the above procedure, ANN model was built for quantitative purposes for determining simultaneously the APIs from their mixture. All the steps involved to carry out the entire work is schematically represented in figure 3.3.

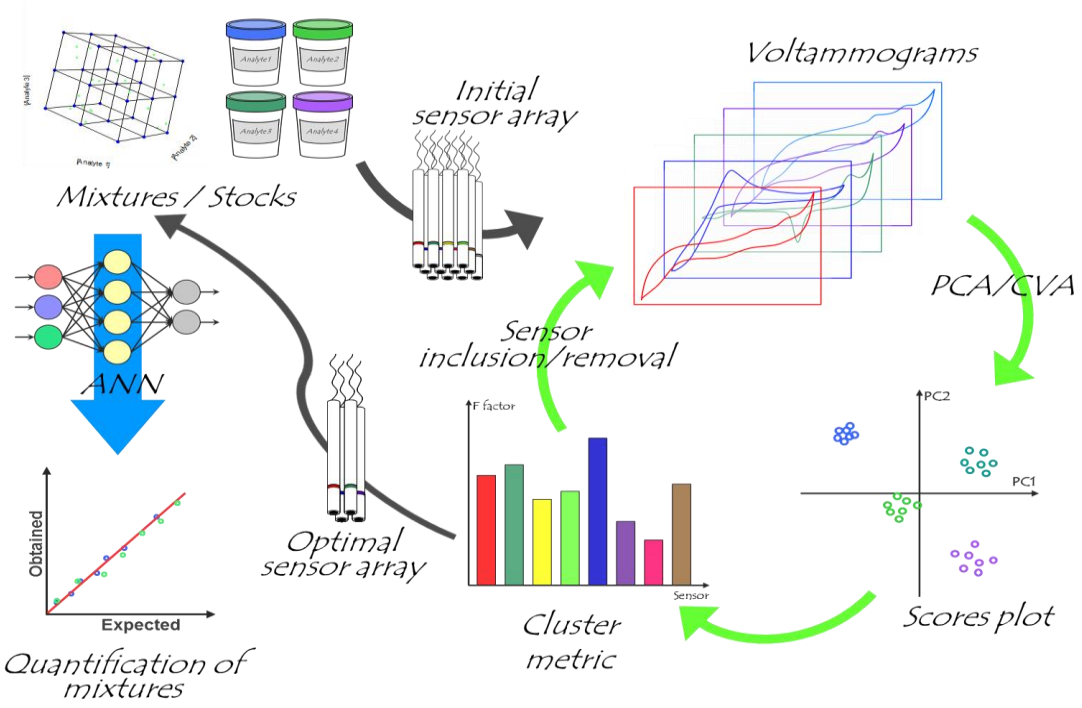

Figure 3.3: Schematic representation of the methodology followed for the a priori selection of the optimal sensor array. Briefly, stock solutions of each of the analytes are measured with all the considered sensors, obtaining a voltammogram for each of them. Next, those are submitted to PCA/CVA, and the clustering is evaluated by means of the F factor. This is repeated, leaving out of the analysis each of the sensors of the array (one at a time), and the one that leads to the higher improvement is removed. The whole process is repeated until a decrease in the F factor is observed after discarding one of the sensors. Finally, with the selected sensor array, the quantitative application is carried out.

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4. RESULTS AND DISCUSSION

4. Results and discussion

Similar to the experimental section, this chapter explains the results related to the two study case considered in this research work.

Study case 1: Determination of capsaicin using a TiO_2 nanoparticles modified electrode.

Study case 2: Determination of Optimal composition of the sensor array of a voltammetric electronic tongue deduced by evaluating the obtained clustering metric

Results obtained for both the study cases are discussed in the following sections.

4.1: Study case 1:

Determination of capsaicin using a TiO2 nanoparticles modified electrode.

As explained in the experimental section 3.4, this work was to verify the applicability of a modified composite electrode for improved sensing of the lipophilic alkaloid capsaicin (CA). Research was carried out to find a better modifier for the improvement of the sensing performance of composite electrode in capsaicin determination. After a screening with different modifiers, redox catalysts and metal oxides, nanoparticles of titanium dioxide (TiO_2) were found to be effective in this study. Results associated with this study case are described in the following sections.

4.1.1. Voltammogram obtained from electrochemical measurements of capsaicin with TiO_2 modified electrode

Redox reactions of capsaicin that takes place in the three-electrode cell arrangement during electrochemical measurements are shown in figure 4.1. For electrochemical characterization of the surface of the TiO_2 modified GEC, capsaicin solution was prepared in the concentration of 60 µM in glycine buffer and cyclic voltammetric measurements were performed. Procedure for the measurements is explained in section 3.4. Voltammograms obtained for CA after 2nd scan of measurements are given in the figure 4.2. As can be seen in the figure 4.2 that there are formations of three voltammetric peaks: two oxidation peaks and a reduction peak. Position of the 1st oxidation peak was at +0.17 and the 2nd oxidation peak was at +0.43 V; the reduction peak was obtained at -0.05 V. Actually the peak at +0.17 wasn't observed during 1st voltammetric scan. Hence the redox system formed during 1st scan in case of capsaicin is related to the oxidation product that formed during the initial oxidation of the pure compound that is in the formation of the 1st peak. Hence his observation signifies the formation of a more easily oxidized intermediate product on the electrode surface with respect to CA during 1st scan.

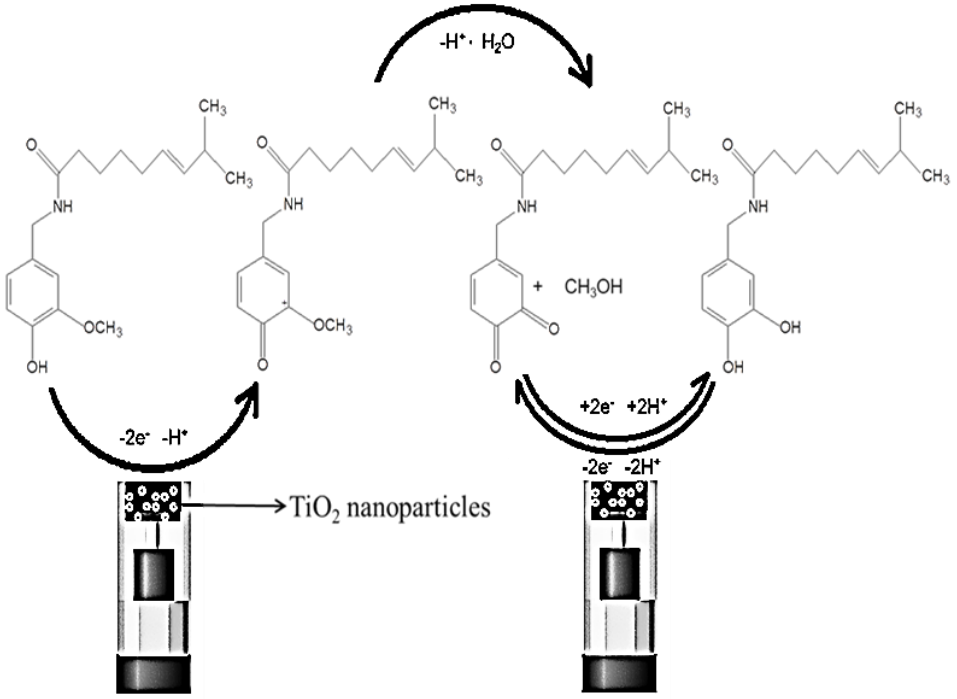


Figure 4.1: Schematic representation of electrochemical oxidation/reduction reaction of capsaicin.

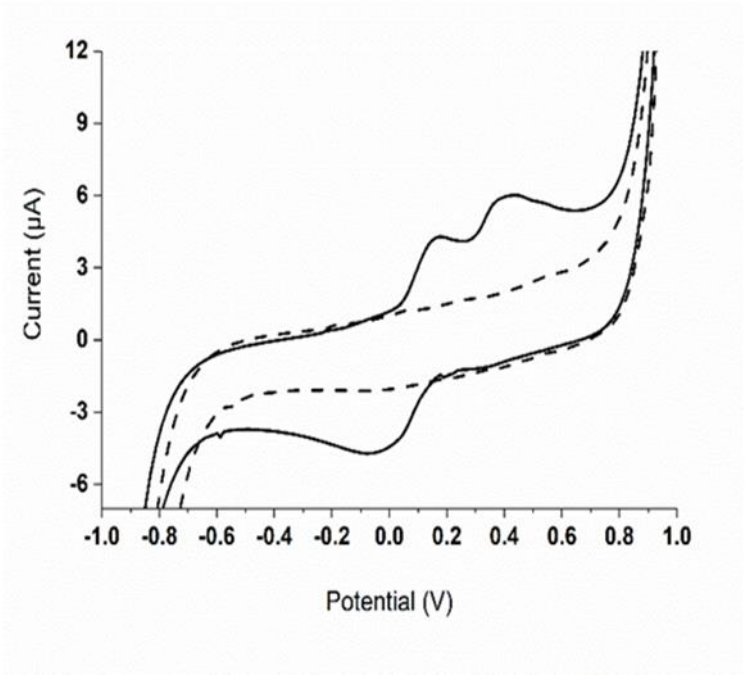


Figure 4.2: Solid line depicts the voltammetric signal obtained after the 2nd scan of measuring capsaicin TiO_2 modified sensor. Dashed line indicates the background buffer.

When the oxidation peaks obtained from this TiO_2 modified sensor were compared with the peaks obtained from other different sensors that are available in the literature (Table 4.1), it is noticed that the peaks are obtained in a lower potential. Therefore, there must be some catalytic effect of TiO_2 nanoparticles that are present in our modified electrode, because of which electrochemical reactions of capsaicin become easier and needs lower energy to take place.

Table 4.1: Comparisons of positions of oxidation peaks of capsaicin obtained from different sensors.

Working electrode	1st oxidation peak (V)	2nd oxidation peak (V)	Reference electrode	Ref
TiO_2 nanoparticles modified graphite epoxy resin electrode	0.17	0.43	Ag/AgCl	This work
Amino-functionalized mesoporosus silica	0.38	0.6	SCE	[1]
Carbon paste electrodes modified by β-cyclodextrin	0.6	0.75	Ag/AgCl	[2]
Screen printed electrode based on multi walled carbon nanotubes	0.5	0.75	SCE	[3]
Diamond electrode doped with boron	0.5	0.75	Ag/AgCl	[4]

4.1.2. Morphological characterization of the modified electrode

In order to study the morphology of the surface of the modified GEC, scanning electron microscopy (SEM) and Energy Dispersive X-ray (EDX) spectroscopy had been used. SEM and EDX images are shown in figure 4.3 and 4.4 respectively.

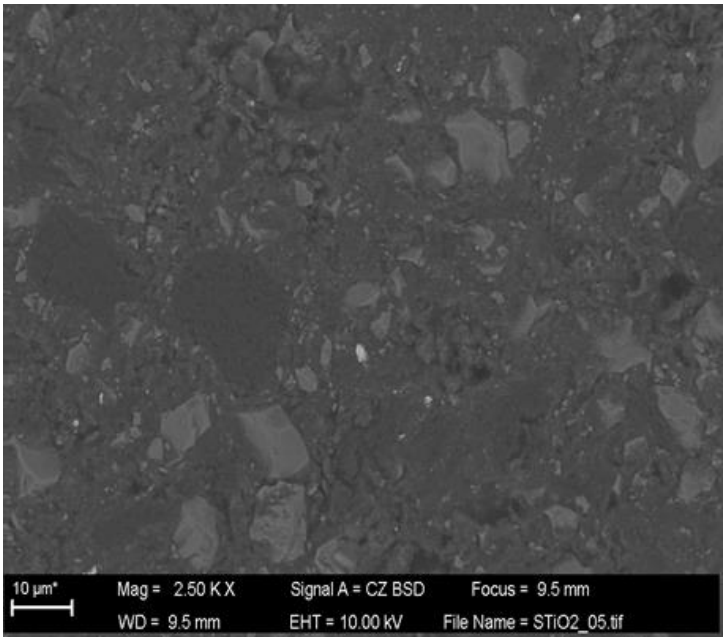


Figure 4.3: SEM image of TiO_2 modified GEC.

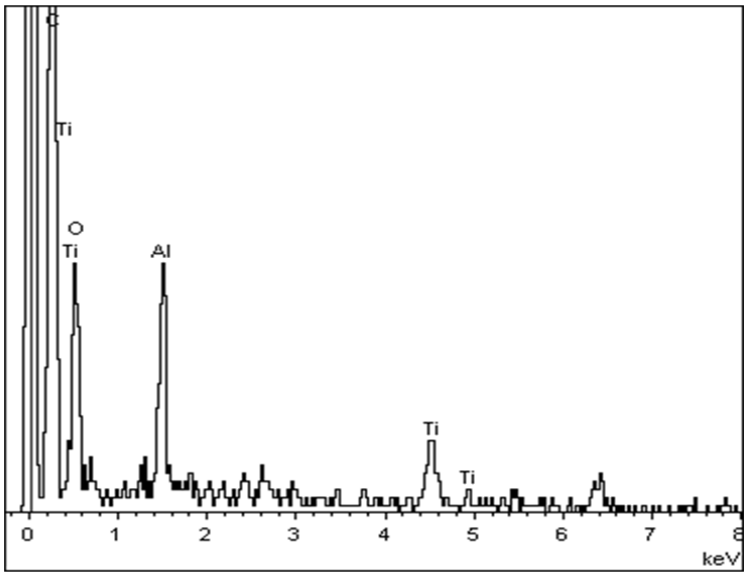


Figure 4.4: EDX study of TiO_2 modified GEC.

4.1.3. pH study for the determination of capsaicin

pH study as explained in the experimental section 3.4 was for determining the most favorable pH for the further characteristic study of capsaicin using the TiO_2 modified sensor. Figure 4.5(A) shows the voltammograms obtained after 2nd scan of measuring capsaicin in the concentration of 40 µM at different pH value, varying from pH 2 to pH 6. From the peak potential of the 1st oxidation peak obtained in each pH, figure 4.5(B) was plotted with potential vs. pH value. Similarly figure 4.5(C) was plotted by taking the value of 1st oxidation peak current vs. pH. It is noticed from the figure 4.5(C) that oxidation peak potential changes linearly with increasing change in the pH. In addition to this, positions of the peak potentials of oxidation peak shifts to the negative potential by 51 mV with increasing per unit pH value . Regression equation obtained was Ep= -0.051pH + 0.323, R^2=0.99. This implies the participation of protons in the electrochemical process. Value of the slope from the equation is found to be near to the theoretical value of 59 mV•pH^{-1}. This signifies that number of electrons and protons exchanged throughout the redox reactions of capsaicin are equal. Moreover, figure 4.5(C) depicts that there is a linear decrease in the oxidation peak current with increase of pH from pH 2 to 6. Therefore, in order to prevent the electrode surface from extreme acidic condition, pH of 2.5 (accomplishable with glycine buffer) was chosen for the successive electrochemical measurements.

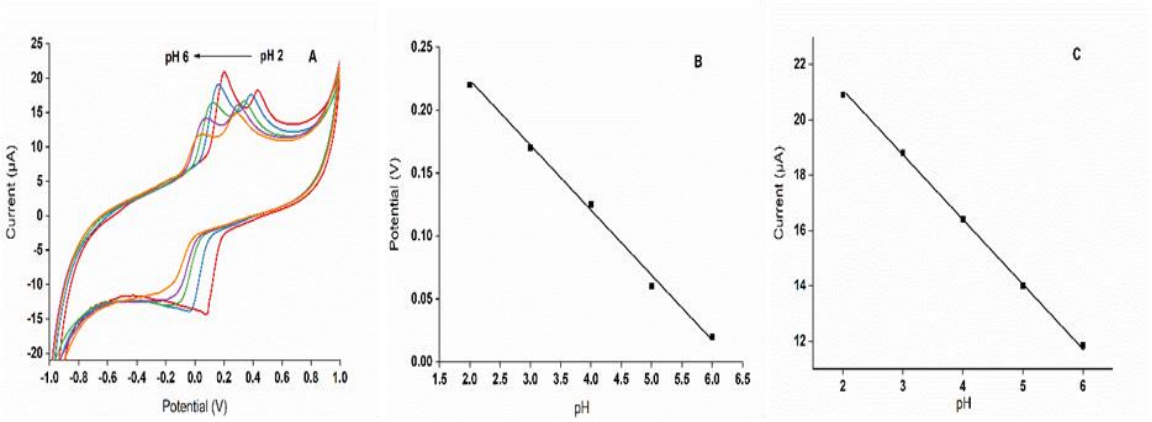


Figure 4.5: Current Vs. Potential curve for 40 µM capsaicin at different pH of the Britton Robinson buffer solution (A), Potential of the 1st oxidation peak vs. pH curve (B), Maximum current for the 1st oxidation peak vs. pH curve (C).

4.1.4. Characterization of analytical properties

A calibration trial was executed in 0.2 mol·l^{-1} in buffer solution of glycine along with 0.1 mol·l^{-1} KCl saline as supporting electrolyte to find out the analytical performance of the electrode. Figure 4.6(A) depicts the voltammetric behavior as attained with the incorporation of Capsaicin to the buffer. For each of the two anodic peaks, two separate calibration curves, of peak current versus CA concentration were plotted. Calibration lines obtained from both the oxidation peaks are found to be linear within a specific concentration range of capsaicin. For oxidation peak at 0.17 V (Figure 4.6(B)), and +0.43 V (Figure 4.6(C)), the linear characteristics are obtained by varying the concentration from 6 to 75 µM and 12 to 138 µM; respectively. The corresponding regression equations are I(µA) =0.044(±0.0021)·C(µM) + 1.63(±0.11) with correlation coefficient 0.993 (n=12) and I(µA) =0.075(±0.0037)·C(µM) + 1.64(±0.31) with correlation coefficient 0.996 (n=17). Detection Limit (LOD) values were estimated by using the following formula

$$LOD = 3.3S_{y/x}/m$$

Here, $S_{y/x}$ is the slandered error of the y value for individual x in a regression line and m is the slope of the regression line. The respective LOD for the first (+0.17V) and second oxidation peak (+0.43V) are found to be 5.34 and 11.3 µM; respectively.

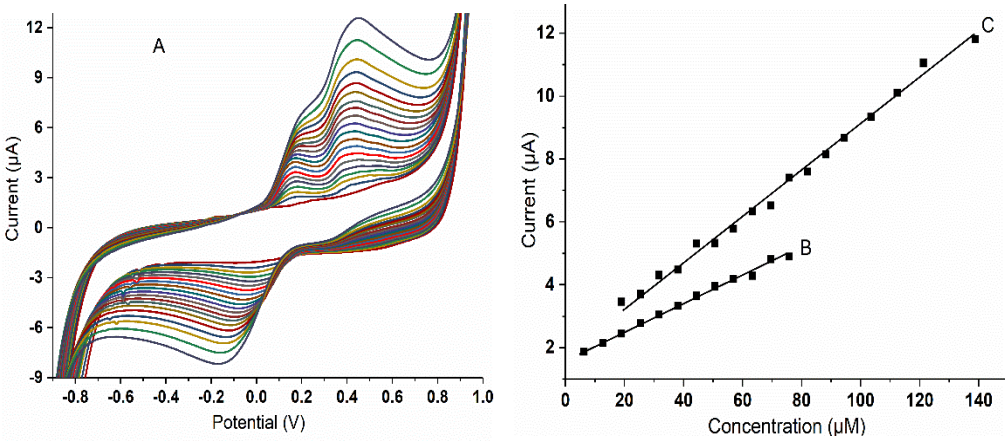


Figure 4.6: Cyclic voltammograms in glycine buffer, pH 2.5 at increasing concentration of capsaicin, (6-138 µM) (A); Calibration line of capsaicin for the 1st oxidation peak (+0.17 V), (B); Calibration line of capsaicin for the 2nd oxidation peak (+0.43 V), (C).

A number of ten cyclic voltammetric measurements of capsaicin with concentration of 23.33 µM in glycine buffer have been performed for checking out the repeatability of the TiO_2 modified electrode. The experimental conditions were followed as described in section 3.4.3. An RSD value of 2.5% was obtained indicating a favorable repeatability of our developed electrode for capsaicin determination.

From the stability study, the value of RSD for solutions of blank buffer was obtained as 2.31%. The corresponding value for capsaicin sample was 3.41%. The RSD values of 9.5% and 14.3% for the respective measurements using unmodified GEC electrode were obtained even using the same rinsing procedure. The difference between the RSD values of unmodified and TiO_2 modified GEC electrode shows prominent prevention of the fouling effect with respect to the unmodified electrochemical determination of capsaicin.

Figure 4.7 indicates a good stability response of our modified GEC electrode TiO_2 nanoparticle during capsaicin measurements. According to the information we have, this is the first research work to check the fouling effect caused by capsaicin. Therefore, with this easy cleaning step and our developed modified electrode, we can minimize the fouling effect associated with the capsaicin measurements. As shown in the figure 4.7(A), we can have stable blank measurement series with no memory effect and hence series of capsaicin measurements with controlled variability can be carried out.

Additionally, RSD values of 3.27% and 3.19% were obtained for reproducibility of polishing with respect to TiO_2 nanoparticles modified GEC and unmodified electrode, respectively, while measuring capsaicin. This signifies that the RSD values are similar both for polishing as well as cleaning with water and ethanol mixture for the TiO_2 modified electrode. This behavior was not observed for unmodified GEC electrode. Therefore, the capsaicin measurement can be done by cleaning procedure only, rather than the time consumed polishing one by our developed sensor. Table 4.1 shows that the RSD values of repeatability measurement in our case are lowest as compared to the other previously reported sensors.

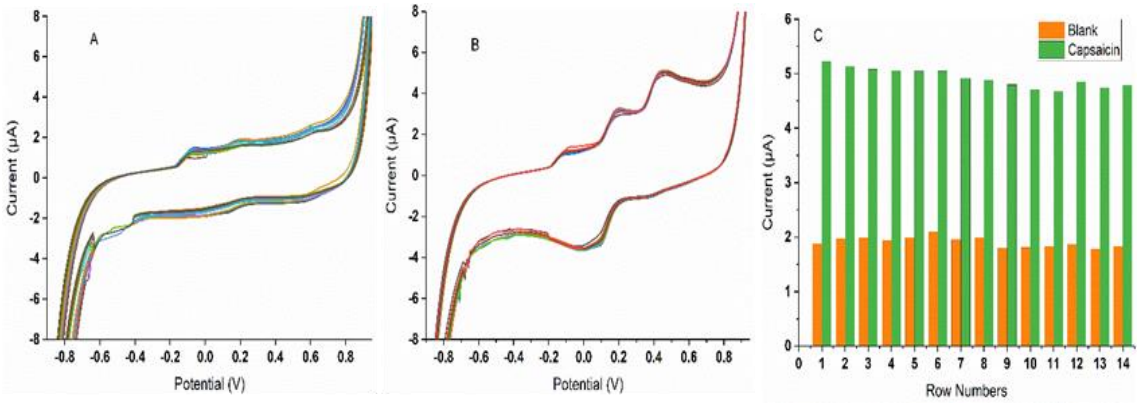


Figure 4.7: Current Vs. Potential curves for repetitive measurements of blank after each cycle (A), repetitive measurements of 35µM capsaicin in a buffer/capsaicin cycle manner (B), comparisons of blank and capsaicin current measurements at +0.43V (C).

Table 4.1: Comparison of analytical properties of different electrodes in the literature used for the voltammetric determination of capsaicin.

Working electrode	Electrochemical technique [(a)]	pH media	Preconcentration time	LOD (μM)	Linearity range μmol L^{-1}	(RSD) %	Ref
Multi walled carbon nanotubes based screen printed electrode	CV	1.0	60 s	0.45	0.5–35	–	[3]
Boron doped diamond electrode	SW	1.0	90 s	0.039	0.16–20	4.44	[4]
Amino-functionalized mesoporosus silica	LSV	3.0	180 s	0.02	0.040–0.4, 0.4–4	–	[1]
Graphite pencil electrodes	ASV	9.0	120 s	0.0037	0.016–0.32	7.1	[5]
Mesoporous cellular foams	DPV	1.0	60 s	0.08	0.76–11.65	–	[6]
Ag/Ag_2O poly(sodium4-styrensulfonate) reduced graphene	DPV	1	60 s	0.4	1–60	9.70(*1)	[7]
Graphene-titania-nafion	LSV	1	10 min	0.0086	0.03–10	–	[8]
Carbon nanotubes and ruthenium nanoparticles	SW	4	–	0.0025	0.01–0.4	5(*2)	[9]
Graphite pencil electrodes	CV	9	–	0.1	0.1–100	–	[10]
Carbon paste electrodes modified by β-cyclodextrin	CV	1 $mol \cdot l^{-1}$ $HClO_4$	–	0.065	1.44–33	–	[2]
Graphite epoxy resin electrode with TiO_2 nanoparticles	CV	2.5	–	5.34	5.34-138	2.53	this work

[(a)] Cyclic voltammetry (CV), linear sweep voltammetry (LSV), adsorptive stripping voltammetry (ASV), differential pulse voltammetry (DPV), square wave voltammetry (SW),

(*1) peak current decreased by 9.70% after five hundred times determination.

(*2) peak current changes by less than 5% after measuring two weeks

4.1.5. Analysis of real sample

As described in experimental 3.4.3, extracted diluted solutions of capsaicin (CA) containing real samples were taken to check the applicability of the TiO_2 modified GEC electrode. This is followed by determination of actual concentration of the CA in the chosen samples. This involves recording of voltammograms after each sample while amounts of capsaicin standard solution were added by gradually increasing the concentration. Figure 4.8 depicts that the behavior (shape, size) of the voltammograms is different in case of extracted and pure capsaicin samples. The observed difference is may be due to the presence of interfering agents in the extracted sample. To overcome this problem, OriginPro 2017 software was used for the baseline corrections of the voltammograms. Concentration of the CA was estimated by considering oxidation peak at +0.43 V. This peak was considered because of the clarity of the voltammograms related to this peak. The voltammograms of real sample Delhuerto hot pepper sauce is shown in Figure 4.8(A). The corresponding voltammograms for Tabasco hot pepper sauce and Alacapsin are shown in Figure 4.8(B) and 4.8(C); respectively [11]. The curves obtained from standard addition of CA for each sample are shown in insets in the respective figure. Each real sample was measured in three replicates to find out the average concentrations of capsaicin (Table 4.2).

Table 4.2: Results of calculated concentrations of capsaicin in different real samples

Pepper samples	Amount of capsaicin determined (μM)	Confidence level (95%)	Actual amount of capsaicin
Delhuerto hot pepper sauce	15.59	±1.49	-
Tabasco habanero pepper sauce	24.68	±2.12	-
Alacapsin	29.06	±2.44	30.7

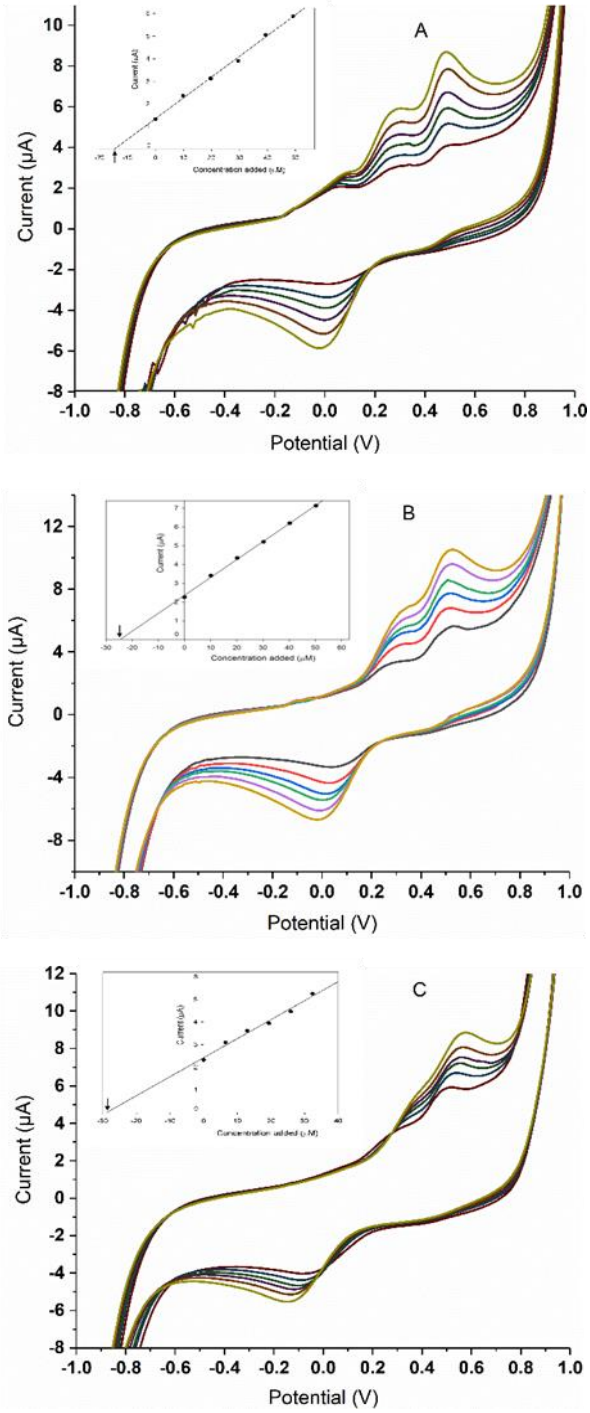


Figure 4.8: Cyclic voltammetric signals for additions of 10, 20, 30, 40 and 50 µM capsaicin on extracted Delhuerto hot pepper sauce (A); 10, 20, 30, 40 and 50 µM capsaicin on extracted Tabasco habanero pepper sauce (B); 6.5, 13, 19.5,26, and 32.5 µM capsaicin on the extracted Alacapsin. (C); All the measurements were carried out in glycine buffer solution (25% EtOH) pH 2.5 at open circuit condition, $\upsilon = 100\ mV \cdot s^{-1}$. The first scans show the signal of diluted real sample before the additions of capsaicin.

4.2: Study case 2:

Determination of Optimal composition of the sensor array of a voltammetric electronic tongue deduced by evaluating the obtained clustering metric.

As explained in the experimental section 3.4 in this research case, eight different kinds of epoxy graphite electrodes, fabricated employing different modifiers (metal nanoparticles, oxide nanoparticles, redox catalysts and conducting polymers_) were first considered. Using canonical variate analysis, principal component analysis and a clustering factor "F", a number of optimum sensors out of 8 sensors were selected for the formation of an electronic tongue system. Sensors that were initially considered for investigating their applicability to form an ET are 7 modified graphite epoxy composite sensors/electrodes (GEC electrodes) and a metallic electrode of platinum. Each GEC electrode was separately modified with, Prussian blue, Polypyrrole, Cobalt (II) phthalocyanine and oxide nanoparticles of titanium (TiO_2), bismuth (Bi_2O_3), zinc (ZnO) and tin (SnO_2). After choosing the optimum sensors for the ET, it was then verified for resolving sample mixtures containing 3 active pharmaceutical ingredients (APIs) including ascorbic acid, paracetamol and uric acid in their different concentrations. Results associated with this study case are described in the following sections.

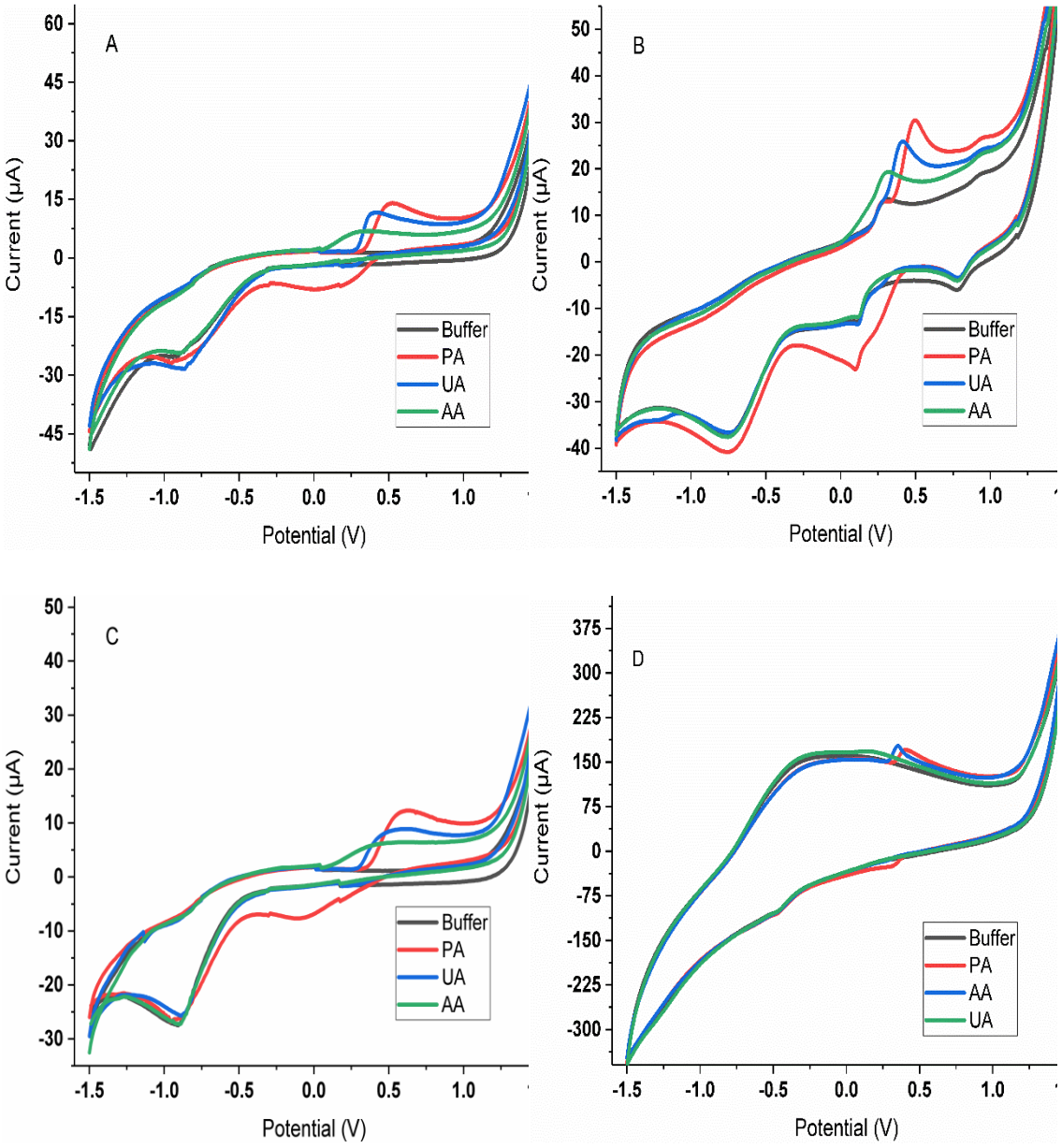
4.2.1. Selection of optimal sensors

While started the selection procedure it was kept in mind that if it is possible to choose the best sensors by measuring a particular set of analytes for only one time then this selection will lead to a better ET model for resolving mixtures of that particular set of analytes.

Therefore, we have considered three analytes, PA, AA and UA as our study case and tried to select the most suitable sensor for resolving mixtures containing these three analytes. During the selection of the optimal sensors, the importance of cross-responses among various sensors as well as towards the set of analytes considered to be analyzed in the ET should be noted. Every electrode response to each analyte in a different way. Moreover, different electrodes depict unique electrochemical responses amongst them. These helps in resolving the different analytes from their mixtures [12].

First of all, it was necessary to assess the electrochemical responses of every sensor for the individual analytes. Hence, voltammetric measurements of the solutions having concentration of 250 µM for each APIs (five replicate) were evaluated separately as explained in Section 3.5.1. Figure 4.9 illustrates the voltammetric

behaviors that were obtained by measuring with different sensors. Different voltammetric behavior was observed with respect to different sensors. This signifies distinct electrochemical responses for individual APIs, a necessary condition to develop electronic tongue applications.



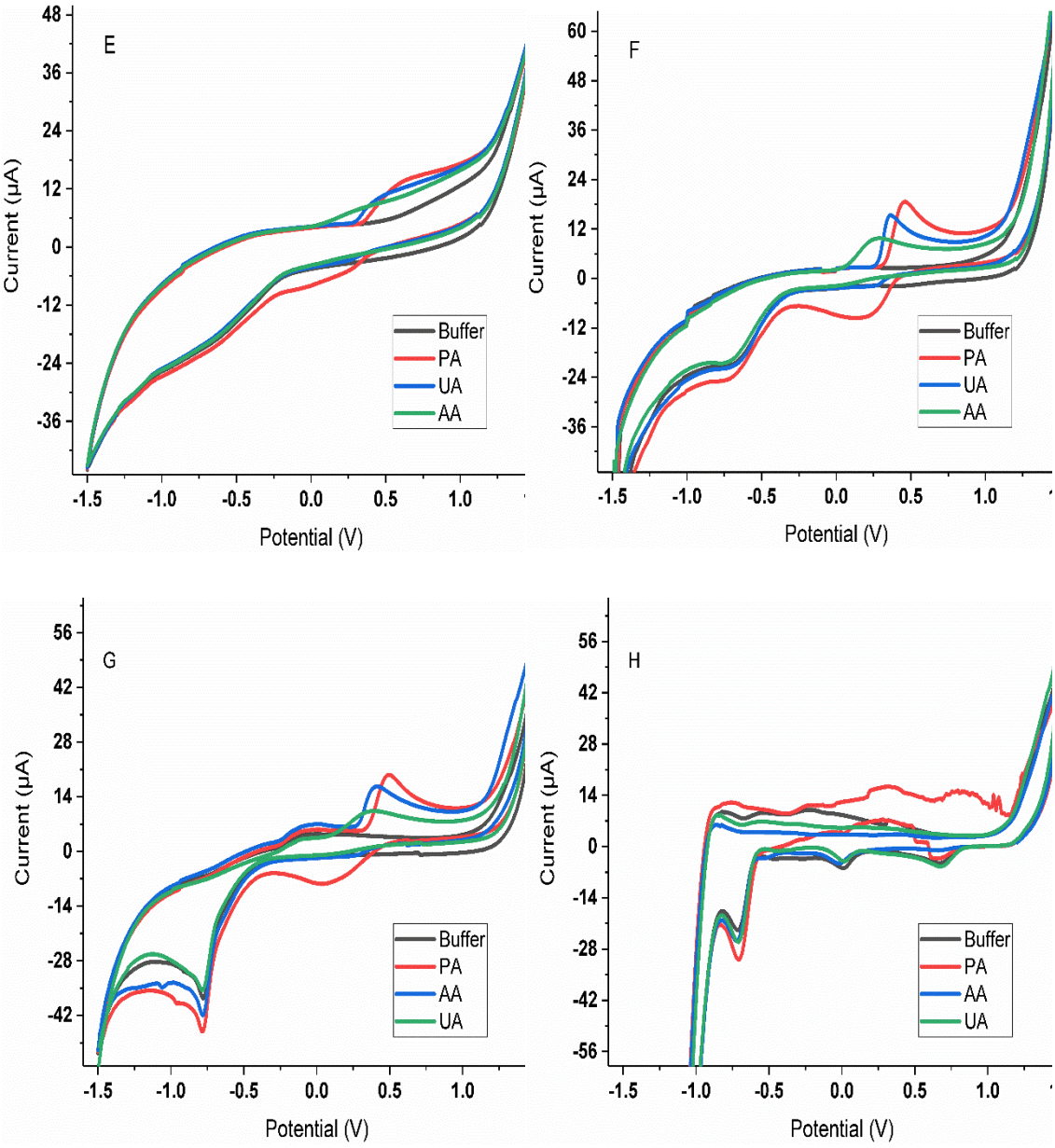


Figure 4.9: Voltammograms obtained for the three APIs (250 µM in phosphate buffer) using the GECs modified with (A) SnO_2, (B) Prussian Blue, (C) ZnO, (D) PPy, (E) CoPc, (F) TiO_2 and (G) Bi_2O_3, and (H) the metallic Pt electrode.

In the next step, data obtained from the above voltammetric measurement were compressed with the help of discrete wavelet transform. The compressed data was then analyzed by CVA and PCA. CVA and PCA provided the score plots (2D) as shown in Figure 4.11(A). and from the score plots the clustering factor, F was determined. This procedure was performed by using the 8 modified sensor array and was continuously

repeated by elimination of one electrode at an instant, similar to leave-one-out method; however, from the electrode' side. In this way, F values were calculated separately for different combinations after removing one electrode at a time. The calculated F values were compared and the sensor was discarded from the initial 8 sensor array at a time if exclusion of it from the 8 sensor set leads to the highest increased value of F. The initially considered electrodes were reduced one after another at a time by following the above procedure repeatedly until the value of F decayed (Figure 4.10).

Because of the manifest improvement of the clustering factor F (4.19 to 5.19), the electrode that was first discarded from the initially considered 8 electrodes was SnO_2 nanoparticles modified one. In the next repeated procedure, it was observed that the removal of electrode modified with Bi_2O_3 nanoparticle leads to a clear increase of F value. Therefore, this electrode was chosen to be the next sensor for discursion from the seven sensors. Moreover, because of no change of F value (increase or decrease) after the removal of TiO_2 modified electrode, this electrode was discarded in the next repeated cycle. Hence, it was assumed that discursion or inclusion of TiO_2 modified electrode has no influence on the overall efficiency of the sensor system. The next iteration method involves the removal of electrode modified with cobalt(II) phtalocyanine. On the other hand, in the next iteration cycle no additional sensors were discarded since the removal of the one having a greater F parameter did not overall improve the F value that was obtained from the previously selected 4 sensors.

With a highest possible F value of 5.75, 3 composite electrodes (GEC) modified with polypyrrole, Prussian blue and ZnO nanoparticles as well as a metallic sensor (Pt) constituted the optimal sensor array of the ET in our study.

Figure 4.11(B) depicts the score plot of PCA obtained by using the selected array of 4 sensors. A high value of an accumulated explained variance of ca. 82.3 % signifies how the majority of the variance can be summarized in only two (PCs) coordinates (from the hundreds dimension of the original vector signal). Moreover, it was observed that clusters obtained from every APIs clearly visible as well as they are easily distinguishable among them. Therefore, from the score plot of PCA and voltammograms, it is clear that in the process of electrochemical resolving of the 3 APIs, calculation of F factor may be a suitable approach in the initial selection of electrodes. In contrast, Figure 11(A) shows the score plot of PCA obtained from

initially considered array of 8 sensors, and here the clusters corresponding to the ascorbic acid, paracetamol and uric acid are not distinguishable.

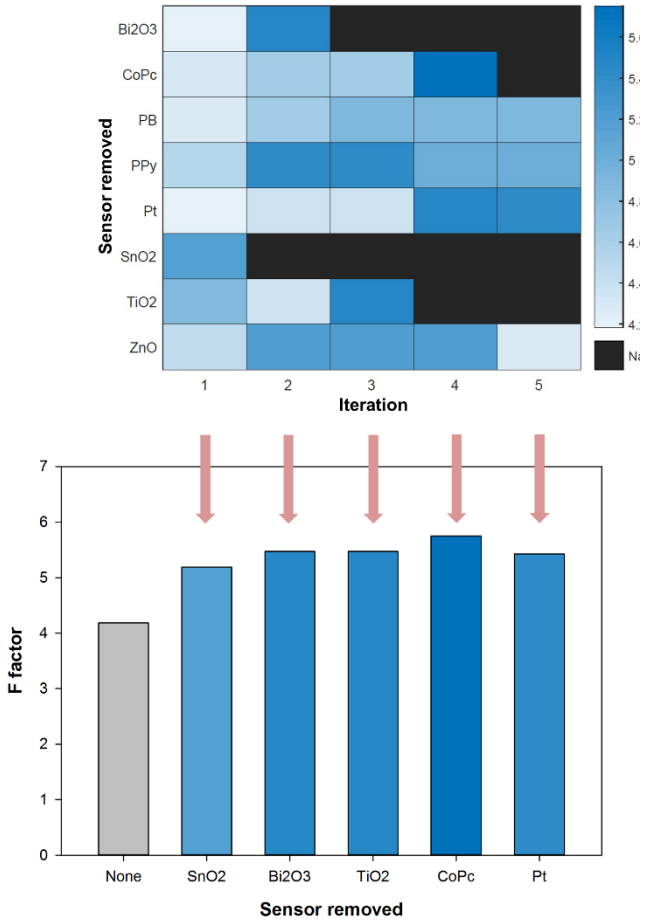


Figure 4.10: (Top) Color map of the variation of the F values after iterative exclusion of the different electrodes; the Y-axis shows the sensor being left out for the calculation of the F factor and the X-axis shows the successive iterations for the selection of the less significant sensor. (Bottom) Bar plot of the changes of the F values after exclusion of the sensor that leads to the biggest F value at each iteration. In both cases, the color of the plot codifies the F factor values as per the color bar. Iteration 5 does not produce any further improvement, and then the process is stopped.

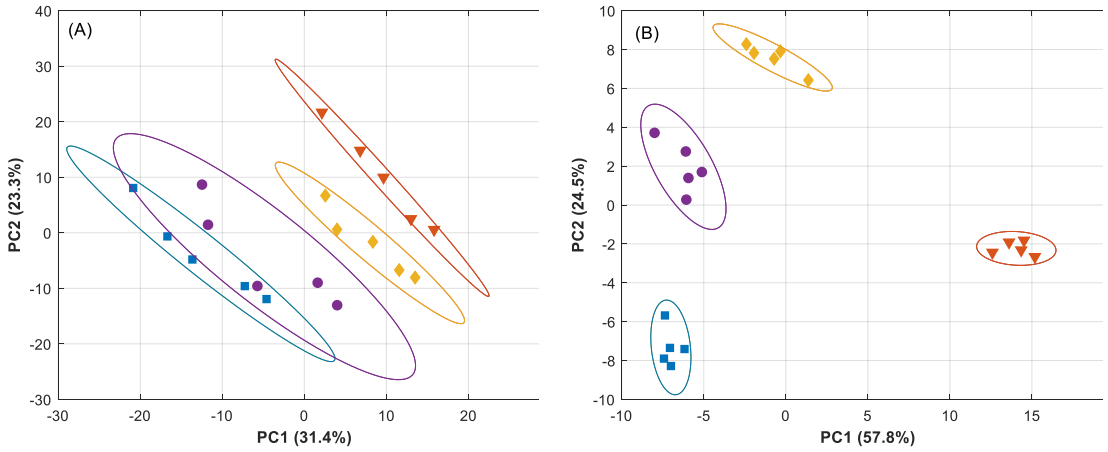


Figure 4.11: .Score plot obtained from the DWT-PCA of five replicas of individual APIs using (**A**) the eight-sensor array or (**B**) the selected four-sensor array. (■) Buffer, (▼) paracetamol, (●) ascorbic acid and (♦) uric acid. Ellipses plotted correspond to 95% confidence limits for each of the clusters.

4.2.2. Characterization of analytical responses

After selecting the optimal sensor array, electrochemical responses of all the four selected sensors towards each of the APIs were characterized. From the characterization, our interest was to verify if all the selected sensors have different sensitivity towards the analytes. As well as we wanted to find out the linearity range for the restriction of the experimental working domain to be considered in the final ET experiment. Additionally, as many numbers of consecutive voltammetric measurements are necessary to work with ET, therefore we evaluated the electrodes' repeatability which is a critical parameter.

Therefore, to evaluate the linearity range of individual APIs with respect to all the selected sensors measurement of APIs with increasing concentrations were carried out. Individual calibration curves were built for each of the selected four sensors towards each of the APIs. For doing this, sample solutions of increasing strength in the range of 0–500 µM were prepared for uric acid and paracetamol. Similarly, ascorbic acid solutions were prepared by taking concentration range of 0-2000 µM. Electrochemical measurements were then carried out as elaborated in 3.5.1. The current for the highest peak was then evaluated from the voltammograms and then plotted with

respect to the corresponding concentration. Table 4.3 summarizes the regression equations obtained from all the compounds. It is observed that the values of coefficient (R^2) are large enough signifying a good linearity within the considered ranges each of the sensors and APIs. Therefore, above mentioned ranges were considered in the final ET model. Additionally, we can conclude that, while measuring our considered analytes all the sensors show different sensitivities (slopes of calibration curves) i.e. all the selected sensors have discrimination ability for the different analytes.

Table 4.3: Calibration data (y vs. x) for the individual calibrations of paracetamol, ascorbic acid, and uric acid employing the final sensors in the array.

Electrode modifier		ZnO	Prussian Blue	Polypyrrole	Metallic Pt
Paracetamol	Equation	y = 0.0598x + 0.613	y = 0.0629x + 10.2	y = 0.0857x + 29.4	y = 0.0027x + 1.08
	R^2	0.9971	0.9993	0.9989	0.9589
	LOD[1] (μM)	28.4	13.9	17.2	59.4
Ascorbic acid	Equation	y = 0.0152x + 0.989	y = 0.0261x + 6.93	y = 0.0304x + 21.0	y = 0.0015x + 0.634
	R^2	0.9977	0.9990	0.9987	0.9624
	LOD[1] (μM)	59.3	68.5	77.5	417
Uric acid	Equation	y = 0.0452x + 1.12	y = 0.0499x + 10.8	y = 0.0668x + 31.0	y = 0.0028x + 1.02
	R^2	0.9988	0.9983	0.9995	0.9967
	LOD[1] (μM)	17.9	23.5	9.35	28.3

Similarly, stability of sensors was checked for all the selected electrodes. Since the final quantitative ET model involves a substantial number of successive measurements by using the electrode array, it is essential to assess if all the sensors show a good repeatability while carried out successive measurements. Therefore, to check the repeatability of the sensors, 100 μM solutions for each of the APIs had been prepared and 18 numbers of successive measurements were carried out without changing any analyst, the equipment and the sample. This was followed by the calculation of relative standard deviation. Voltammetric measurement of a blank buffer solution was carried out before every sample measurement in a cyclic manner. In this way, we tried to ensure that there would not be any change of signal while measuring with mixtures of samples during ET study. The blank buffer solution measurements

helped us to confirm that there was no drifting of the baseline. Electrochemical cleaning of the surface of the electrodes was performed by recording a voltammogram in phosphate buffer after each cycle measurement. RSD values obtained for the electrodes were found to be less than 4% in every case, which indicates a satisfactory stability of the sensors. The RSD% obtained for different electrodes during 18 successive cycles were 2.4%, 1.99%, 2.4% and 3.6% for the electrodes modified with ZnO, PB, PPy and Pt sensor; respectively.

4.2.3. Quantitative study of APIs Mixtures

After the selection of the sensors for the study of uric acid, ascorbic acid and paracetamol, the next experimental step was to find out the ability of such sensors array to resolve the analytes from their mixtures. To achieve this, electrochemical measurements of samples as explained in section 3.5.2, was carried out following the same procedure (Figure 4.12). As expected, it was noticed that voltammograms obtained for the sample mixtures are different than that were obtained for individual compound. This different response of the voltammograms is due to the overlapping of signals while measuring the mixtures. As the quantification cannot be achieved through univariate regression, chemometric models are required for the same. Chemometric models can resolve cross response features of the sensors i.e. the ability of different electrodes to sense differently, the individual APIs.

Before using ANN quantification model [13], first of all it is necessary to compress the large dimensionality of the original data. The compressibility of the data is a critical step in an ANN quantification model which prevents problems of under-determination that may arise with an extremely complex set of data related to oversized ANN. Additionally, data compression also helps in reducing memory as well as time while dealing with ANN. On the other hand, as the redundancy within the input data is avoided here along with the reduction of their complex behavior (with over fitting risk), this can lead to models having generalization capability as well as better performance [14]. In our study, the data compression was carried out by DWT, based on "Daubechies wavelet mother function" of fourth order decomposition level. Our original data had 1696 points that were corresponding to currents measured at 424 number of polarization potentials using 4 selected sensors (424×4). DWT helped us to

reduce these data points upto 132 coefficients i.e. by 92.2% without losing any significant information. We obtained fc > 0.95, r > 0.99 while compared the compressed data with respect to the original signal.

For selection of the topology of the neural network, an organized study was performed which involves varying the transfer functions between the layers of hidden and input as well as between the layers of output and hidden. Moreover, in the hidden layer neurons number were also varied.

The data related to the training subset were used for the building of different possible types of ANN models. Then the best ANN model was selected after verifying the ability of resolving the mixtures in the testing subsets. This procedure helped to increase the efficiency of the final model by detection of the possible over fitted models. In the finally selected ANN model, the input layer was constituted by 132 neurons. This corresponded to the coefficient of DWT, 33×4 electrodes. Similarly, the hidden layer was comprised of 6 numbers of neurons along with the transfer function, *purelin.* The output layer had 3 numbers of neurons and transfer function, *satlins.*

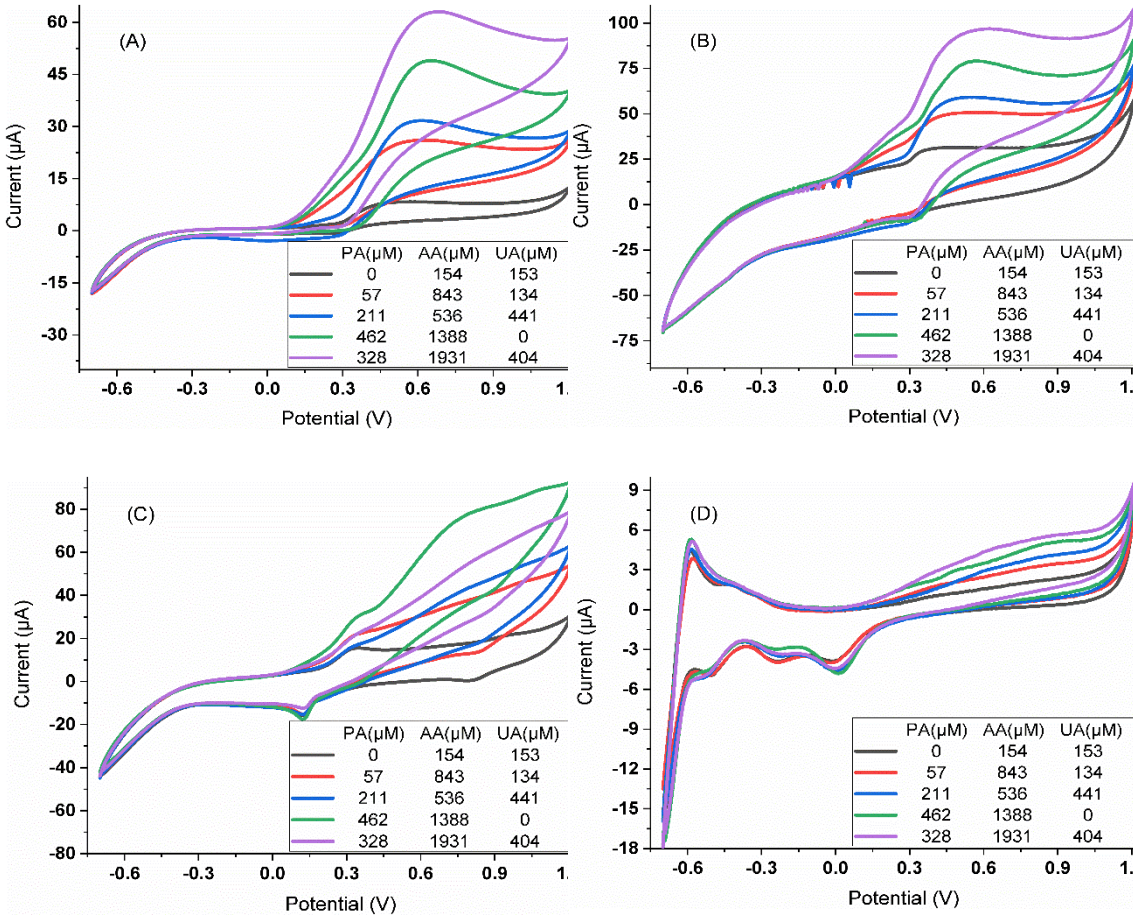


Figure 4.12: Representative voltammograms obtained for certain arbitrary mixtures of the different APIs (the concentration for each compound is indicated in the legend) with the four-sensor selected array: GECs modified with (**A**) ZnO, (**B**) PPy and (**C**) Prussian Blue, and (**D**) the metallic Pt electrode.

For the selected ANN model, the graphs for the comparison of obtained and expected concentration along with fitted liner regression for paracetamol is shown in the figure 4.13(A) Whereas, the figure 4.13(B) and 4.13(C) correspond to the same for ascorbic acid and uric acid; respectively. A satisfactory behavior was observed for each of the analytes, showing a close overlapping of the regression lines with the perfect (y=x) ones. For the better evaluation of the comparison, calculation of parameters such as correlation coefficient, slope, intercept, etc. were carried out (Table 4.4). The parameters (correlation coefficient, slope, intercept) were found to be very close with the ideal ones (1, 1, 0). This signifies that our ANN model shows a good ability for resolving the mixtures of the APIs.

Moreover, to verify that the previous one are within the confidence area of the regression parameters that were calculated, we have determined as well as plotted (Figure 4.14) the joint confidence interval. It helps to find out rapidly any differences that may exist between the predicted and the actual values for a definite significance level, as it judges simultaneously the goodness of the intercept and the slope [15]. As shown in figure, the point at (1, 0) for ideal regression line (y=x) is found to be inside the ellipsoidal confidence area obtained from our analytes for both training (Figure 4.14(A)) as well as testing subset (Figure 4.14(B)). Hence, we can say that the model could predict well actual concentration of the testing subsets.

Though the results are more accurate in case of training subsets than the testing subsets, for both the cases the results are significantly accurate. Two factors can explain the higher confidence level related to the testing one. Firstly, during the modeling step, not a single sample from the testing subsets was used and therefore, representing more practical metric of the performance of the model.

Secondly, as the number of samples in the testing subsets is lower than the training subsets, higher values of *t* and F (tabulated) occurs and consequently higher confidence intervals was obtained. At the same time, larger uncertainty in the intercepts value for ascorbic acid was obtained due to the use of a larger range of concentration (0 to 2000 µM).

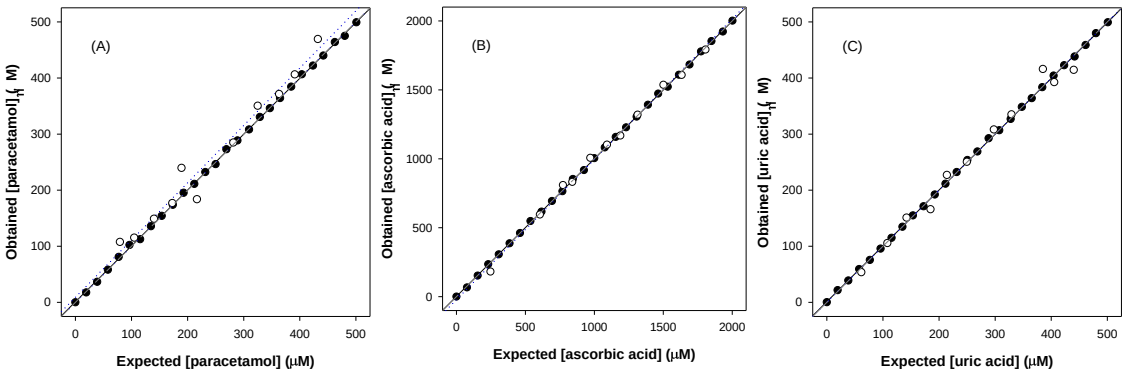


Figure 4.13: Modeling ability of the optimized DWT-ANN. Comparison graphs of obtained vs. expected concentrations for (**A**) paracetamol, (**B**) ascorbic acid, and (**C**) uric acid, for both the training (●, solid line) and testing subsets (○, dotted line). The dashed line corresponds to the ideal comparison line (y =x)

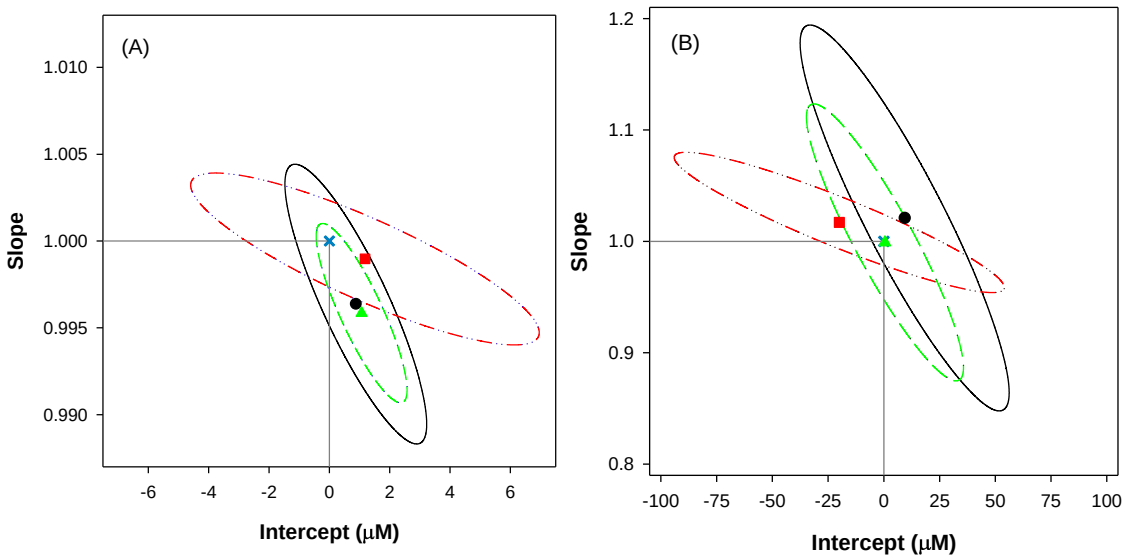


Figure 4.14: Joint confidence intervals for the three species: (●, solid line) paracetamol, (■, dash-dotted line) ascorbic acid, and (▲, dashed line) uric acid, and both for (A) training and (B) testing subsets. Also, the ideal point (1,0) is plotted (x); intervals are calculated at the 95% confidence level.

Finally, the performance of the proposed ET in our study, where electrodes in the sensors array were selected priory by means of CVA, PCA and F factor, was

compared with the previous reports where the same sample matrix were studied (Table 4.4) [REF,]. In order to compare, calculation of parameters such as root mean square error (RMSE) and its normalization (NRMSE) were performed to create a universal metric related to the system performance. Some dissimilarity in the performance may be arised because of the use of different methods for the data treatment (ANNs vs. PLS), however, as we have obtained a significant improvement in the above parameters (RMSE, NRMSE), in the present case, this must be because of the apriori selection of the sensor that we used here. Total NRMSE value was found to be smallest in this work. Additionally, significant improvements of the correlation coefficients as well as the slopes were also obtained [16].

Table 4.4: Reported values and current results of the fitted regression lines for the comparison between obtained vs. expected values for the different sets of samples and the three considered APIs. Intervals are calculated at the 95% confidence level.

Compound	Slope	Intercept (μM)	R^2	RMSE[1] (μM)	Total NRMSE[1]	Sensor array	Ref.
training subset (n = 33)							
Paracetamol	0.942 ± 0.031	32 ± 21	0.968	[2]			
Ascorbic acid	0.933 ± 0.040	36 ± 25	0.947	[2]	[2]		
Uric acid	0.873 ± 0.046	58 ± 25	0.923	[2]		Bare GEC plus metallic Pt and Au electrodes	[17]
testing subset (n = 15)							
Paracetamol	0.895 ± 0.105	82 ± 71	0.848	[2]			
Ascorbic acid	0.919 ± 0.081	65 ± 41	0.908	[2]	[2]		
Uric acid	0.871 ± 0.138	−8 ± 86	0.753	[2]			
training subset (n = 33)							
Paracetamol	0.981 ± 0.032	13 ± 24	0.992	29			
Ascorbic acid	0.990 ± 0.031	6 ± 17	0.993	25	0.0257		
Uric acid	0.981 ± 0.027	9 ± 16	0.994	23		Bare GEC plus metallic Pt and Au electrodes	[18]
testing subset (n = 15)							
Paracetamol	0.990 ± 0.143	−2 ± 80	0.945	97			
Ascorbic acid	1.009 ± 0.136	−28 ± 78	0.952	66	0.101		
Uric acid	0.992 ± 0.208	36 ± 125	0.891	73			
training subset (n = 27)							
Paracetamol	1.000 ± 0.082	0 ± 25	0.962	29			
Ascorbic acid	1.000 ± 0.089	0 ± 25	0.955	31	1.00		
Uric acid	1.000 ± 0.104	0 ± 31	0.940	36		SPCEs modified with CoPc, PB, graphite and CuO	[19]
testing subset (n = 12)							
Paracetamol	1.021 ± 0.219	−13 ± 28	0.915	32			
Ascorbic acid	1.073 ± 0.422	−3 ± 54	0.762	71	1.03		
Uric acid	1.044 ± 0.334	−32 ± 36	0.829	44			
training subset (n = 27)							
Paracetamol	0.996 ± 0.006	0.9 ± 1.9	0.9998	2.43			
Ascorbic acid	0.999 ± 0.004	1.1 ± 4.6	0.9999	5.86	0.00378		
Uric acid	0.996 ± 0.004	1.1 ± 1.2	0.9999	1.64		GECs modified with ZnO, PB, and PPy plus Pt metallic electrode	This work
testing subset (n = 11)							
Paracetamol	1.021 ± 0.134	9 ± 36	0.971	26.4			
Ascorbic acid	1.017 ± 0.049	−20 ± 57	0.996	31.2	0.0368		
Uric acid	0.999 ± 0.096	1 ± 27	0.984	16.2			

[1] RMSE: root mean square error; NRMSE: normalized root mean square error;[2]data not available; GEC: graphite epoxy composite; SPCE: screen printed carbon electrode.

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5. CONCLUSIONS

5. Conclusions

This PhD thesis can be summarized in the following conclusions

1. Modification of graphite epoxy composite electrodes has been performed using 7 different modifiers along with fabrication of a metallic Pt disc electrode. Modifiers considered for the modifications are

    - Titanium((IV) oxide (TiO_2) nanoparticles,
    - Bismuth (III) oxide (Bi_2O_3) nanoparticles,
    - Zinc (II) oxide (ZnO) nanoparticles,
    - Tin (IV) oxide (SnO_2) nanoparticles,
    - Prussian blue (PB),
    - Cobalt (II) phthalocyanine (CoPc) and
    - Polypyrrole (PPy).

    All of the modifiers are found to improve the sensing characteristics as well as able to give differentiated responses for different analytes.

2. TiO_2 nanoparticlesmodified graphite epoxy composite electrode shows improved responses electrochemical determination of capsaicin and therefore it can be an alternative to human-subjective Scoville organoleptic test.

3. Moreover, as far as we know, our TiO_2 modified developed sensor is the first sensor to be fabricated which show very low fouling effect in electrochemical capsaicin determination. Therefore consecutive measurements can be carried out without renewing the surface of the electrode between the measurements.

4. This voltammetric procedure with TiO_2 modified electrode could successfully quantify capsaicin in various real samples including pharmaceutical and hot sauces.

5. In addition to capsaicin determination with the use of single electrochemical sensor, this PhD thesis work also successfully developed a voltammetric electronic tongue for simultaneous quantification of analytes.

6. The sensor array used in the developed electronic tongue were obtained from prior selection of the optimal sensors with the use of canonical variate analysis, principal component analysis and a clustering metric F. PCA and CVA were used to assess the cross-response of the different sensors while the F factor to numerically selects the optimal sensors from the scores plot.

7. To illustrate the applicability of the electronic tongue with this a priory selection methodology, simultaneous quantification and discrimination of three different APIs namely Paracetamol, uric acid and ascorbic acid have been demonstrated. In this study 4 optimal sensors for the ET were selected as the optimal sensor array from 8 initial sensors considered.

8. Next, the performance to carry out the quantitative determination of the three APIs was attempted by building a DWT-ANN model. The performance of the model was very satisfactory, and huge improvement was observed when benchmarked against other previously reported ETs performing the resolution of the same mixtures.

9. This improvement confirms the enhancement of the performance of ETs obtained from the a priori selection procedure.

6. ANNEX

6.1. Publication 1:

Improved Sensing of Capsaicin with TiO2 Nanoparticles Modified Epoxy Graphite Electrode.

Munmi Sarma and Manel del Valle,

Electroanalysis, 2020, 32, 230-237.

Full Paper Wiley Online Library ELECTROANALYSIS

DOI: 10.1002/elan.201900400

Improved Sensing of Capsaicin with TiO_2 Nanoparticles Modified Epoxy Graphite Electrode

Munmi Sarma[a] and Manel del Valle*[a]

Abstract: The presented research focuses on the electrochemical determination of capsaicin, a lipophilic alkaloid which originates hotness in chili peppers. An electrochemical sensor based on epoxy-graphite composite with the modification of titanium dioxide (TiO_2) nanoparticles is developed for the determination of this alkaloid. The measurements were carried out in glycine buffer at pH 2.5 using cyclic voltammetry. Two linear concentration ranges were obtained from 6 to 75 µM (R = 0.99) and from 12 to 138 µM, with a detection limit of 5.34 µM and 11.3 µM capsaicin, for 1st and 2nd oxidation peak, respectively. The main advantage of developed sensor is its repeatability and robustness against fouling; the relative standard deviation (RSD) value was 2.53 % (n = 10). This voltammetric sensing procedure has successfully been applied to quantify capsaicin in various real samples such as hot chili sauce and pharmaceutical preparations.

Keywords: Capsaicin · TiO_2 nanoparticles · hot pepper sauce · analgesic · cyclic voltammetry · cream

1 Introduction

Chili peppers are a highly valued horticultural commodity, used in culinary purposes for adding pungency or hot, spicy flavor to the dishes. The pungency of chili peppers is imparted by the naturally occurring lipophilic alkaloids present, broadly known as capsaicinoids [1–2]. The two major capsaicinoids present in most varieties of hot peppers are capsaicin and dihydrocapsaicin, constituting 90 % or more of the total capsaicinoids [3]. The remaining 10 % are nordihydrocapsaicin, norcapsaicin, homocapsaicin, homodihydrocapsaicin, and nonivamide [4]. Capsaicin is present in large quantities in the placental tissues of chilli peppers that hold the seeds in fruits and may acts as a repellent against herbivores [5]. Chemically, capsaicin is trans-8-methyl-N-vanillyl- 6-nonenamide; it's handbook description is: fat soluble, odourless, pungent tasting, off-white solid with a melting point of 62–65 °C and a molecular weight of 305.4 g mol^{-1}

Besides its usage in food preparations, capsaicin has also application in pharmaceutical industry due to its several pharmacological properties [5–7]. It is well-known to have anti-bacterial [8], anti-carcinogenic [9], anti-tumoral [10], anti-mutagenic [11] properties and it is a good anti-oxidant [12]. Capsaicin is also used as an ingredient in drugs to control obesity [13], cholesterol [14] and in topical formulations used for pain management [15–16]. In addition, capsaicin based pepper sprays are used as tools for skin conditioning, self-defense and also to control mob violence by security authorities [17].

Since its first isolation and determination of its chemical structure in 1919, several advancements have been made in extraction and quantitative estimation of this alkaloid [18]. Its widespread usage calls for capsaicin estimation methods which are simple, fast and accurate. Initial pungency of hot sauces and peppers were estimated by a classical method known as Scoville's organoleptic test [19]; this uses a technique where a solution of pepper extract is diluted in a solution of sugar water until the heat or pungency is no longer sensible by taste. In addition to this traditional semi-quantitave method, a lot of other analytical techniques have been established for estimation of capsaicin. Among these are high performance liquid chromatography (HPLC) [20–22], immunoaffinity chromatography combined with liquid chromatography-tandem mass spectrometry [23–24], enzyme immunoassay [25–28], capillary electrophoresis [29], colorimetric method [30], micellar electrokinetic capillary chromatography [3], HPLC coupled with electrochemical detection [31], photo diode array and mass spectrometry [32], fluorescence spectroscopy [33], spectrophotometry [34–35], etc. Although some of these variants e.g. HPLC coupled with enhanced detection like mass spectrometry give satisfactory results with respect to sensitivity and resolution, these techniques need to face complex procedures which includes expensive instrumentation, long response time, difficulty in sample preparation etc. Therefore, to overcome these difficulties, new techniques need to be established with objectives like less expensive instrumentation, ease of use, portable, favorable sensitivity towards capsaicin, etc. Electrochemical sensors provide an excellent tool for performing on-site analysis at a reasonably cheap price with fast and robust results.

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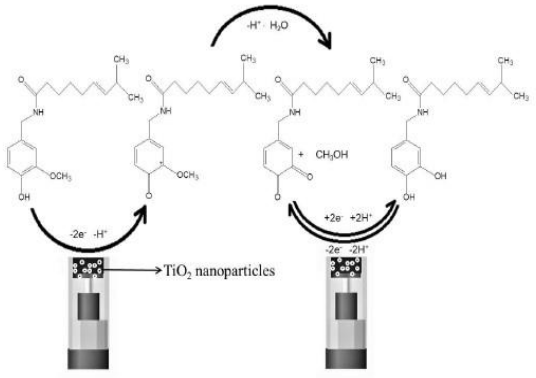
Supporting information for this article is available on the WWW under https://doi.org/10.1002/elan.201900400

Electrochemical analysis seems to be a promising technique for the proper quantitative estimation of capsaicin since Compton et al. first demonstrated a electrochemical technique which involved its voltammetric determination using a carbon nanotube based electrochemical sensor [36]. Later on, extensive researches on electrochemical analysis for capsaicin have been improving the technique day by day. All these research use some modifiers in the electrode to improve the sensing of capsaicin. Variants described include boron-doped diamond electrode [37]; carbon paste electrodes modified with amino-functionalized mesoporous silica [38], mesoporous cellular foams [39], β-cyclodextrin [40]; glassy carbon electrode [41]; glassy carbon electrode modified with graphene-titania-nafion composite film [42], ruthenium nanoparticles decorated carbon nanotubes platform [43], polypyrrole/Bi_2O_3/graphene oxide [44]; graphite pencil electrode [45–46]; single-walled and multi walled carbon nanotubes based screen printed electrode [47–48]; screen printed electrode modified with Ag/Ag_2O nanoparticles/reduced graphene oxide [49]; poly(sodium 4 styrenesulfonate) functionalized graphite [50]; paraffin-impregnated graphite electrode [51]; polyaniline electrode [52]; biosensor based on a horseradish peroxidase enzyme-capsaicin reaction mediated by ferrocene [53]; enzyme biosensor using ammonia-lyase enzyme [54], etc. Mechanistic steps involved in the electrochemical reactions of capsaicin are shown in the Scheme 1 [36].

It is observed that carbon based electrodes lead to fouling effect in the cyclic voltammetric measurements of capsaicin. This fouling results in unstable baselines with decreasing oxidation peak current. In order to overcome this problem, extensive search of electrode modifiers, mainly of nano technological origin, has been made. Metal oxide nanoparticles have been used in the modification of electrochemical sensors from a long time due their interesting sensory properties like functional biocompatibility, chemical stability, controllable size, biosafety and catalytic effects. Along with enhanced electron transfer kinetics, they may also offer adsorptive possibilities for stripping voltammetric variants. Specially, if incorporated to electrodes in nanopowder form, these materials present interesting advantages over other conventional materials on account of electron transfer and immobilization of biomolecules for enhanced chemical and biological sensor operation [55]. Here, in this research, we have developed a sensitive, rapid and efficient electrochemical sensor based on epoxy-graphite composite with the modification of Titanium dioxide (TiO_2) nanoparticles for the determination of capsaicin. TiO_2 nanoparticles were incorporated into the sensor by adding the nanoparticles into the mixture of epoxy graphite composite during the fabrication of the sensor. This developed sensor is then farther used for successful determination of capsaicin content in various hot pepper sauce and pharmaceutical products with enhanced reproducibility features and minimal fouling effect.



Scheme 1. Electrochemical oxidation/reduction reaction of capsaicin.

2 Experimental

2.1 Reagents

All reagents were of analytical reagent grade and were used as received without any further purification. Capsaicin as commercially available was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals for the preparation of the buffers were purchased from Merck (Darmstadt, Germany). Titanium (IV) oxide (< 25 nm) nanoparticles, which were used for the modification of electrodes, were purchased from Sigma-Aldrich. Capsaicin mother solutions were prepared in 48 % ethanol as it is not completely soluble in water. All the buffer solutions were prepared in ultrapure water purified by a MilliQ System (Millipore, Billerica, MA, USA). Graphite powder (particle size < 50 µm) used for the construction of the electrodes was purchased from BDH (BDH Laboratory Supplies, Poole, UK) and the corresponding H77 resin and the hardener were obtained from Epoxy Technologies (Billerica, MA, USA). For the real sample analysis two commercially available sauce samples and a pharmaceutical cream were purchased. These are Tabasco Habanero hot pepper sauce from McIlhenny company (Avery Island, LA, USA), Delhuerto original hot pepper sauce (Peru) and one analgesic cream named "Alacapsin" from Alfasigma company (Spain).

2.2 Apparatus

All the cyclic voltammetric measurements were carried out in a PGSTAT 30 Autolab potentiostat (EcoChemie, The Netherlands) with GPES 4.7 version software (EcoChemie). For the pH adjustments during pH study and preparation of the electrolyte solutions, a Crison micro pH 2002 pH-meter (Crison instruments, Barcelona, Spain) was used. Electrochemical measurements were carried out in a conventional 3 electrode cell arrangement where a combined electrode (Crison 5261, Barcelona, Spain) was used, made up of metallic platinum wire and

Ag/AgCl electrode, as an auxiliary and reference electrode, respectively.

2.3 Construction of Working Electrode

Graphite epoxy composite electrode (GEC) modified with TiO_2 nanoparticles was used as a working electrode. Working electrodes were constructed with the help of a 6 mm i.d. PVC tube [56]. First of all a shaped copper disc is soldered to an electrical connector. This couple is then put into the PVC tube as shown in the Figure 1.A,

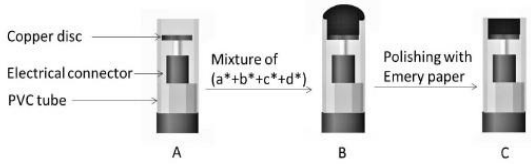


Fig. 1. Graphite-epoxy composite electrode construction. a*: graphite powder, b*: Titanium oxide nanopowder, c*: Epoxy resin d*: Hardener.

resulting in a cylinder cavity. A paste is then made by mixing 54 % graphite powder, 4 % titanium oxide nanopowder and the corresponding epoxy resin and the hardener; this paste is then used to fill the cavity and cured at 40 °C for 48 h. The electrode surface is then polished with emery papers of decreasing size until a flat, shiny surface appears, see Figure 1.C. This prepared electrode is then used as a working electrode for the electrochemical measurements of capsaicin. Whenever needed, the repolishing of the surface regenerates the electrode, recovering any loss of the response.

2.4 Sample and Electrolyte Preparation

The supporting electrolyte solution for preparation of the capsaicin samples and for calibration used in this study was 0.2 mol L^{-1} glycine buffers with 0.1 mol L^{-1} KCl as saline background. Glycine buffer at pH 2.5 with KCl as saline background is selected in this study as a result of of optimization of pH. Britton Robinson buffer with 0.1 mol L^{-1} KCl was prepared for optimization of pH measuring conditions.

2.5 Extraction of Capsaicin from the Real Samples

For the extraction of capsaicin from the sauce samples, specific procedure was used as capsaicin is not totally soluble in water. An aliquot of 2.5 g of sauce is first weighed in a beaker containing 35 mL of 96 % ethanol and then the beaker is sonicated for 15 min and then magnetically stirred for 2 h. This capsaicin extracted solution is then filtered using glass fiber filter paper (GMFA SCHARLAU Ø 90 mm) into a 50 mL volumetric flask; after filtration, the funnel containing the solid part of the sauce is rinsed with some aliquots of ethanol until filling up of the volumetric flask up to the mark. This rinsing procedure was followed so that all amount of capsaicin had been extracted from the remaining solid parts of sauce in the filter. This extracted solution was then used for voltammetric measurements. The same extraction procedure was also followed for the extraction of capsaicin from the pharmaceutical cream sample.

2.6 Analytical Procedure

Complete voltammograms were recorded for capsaicin by cycling the potential between −0.9 V and +1 V vs. Ag/AgCl with a step potential of 0.01 V and a scan rate of 100 mV s^{-1}. After each measurement chemical cleaning of the electrode surface was carried out; this involves rinsing the surface in specific media (48 % ethanol: water, v/v) for 60 seconds with stirring. For the voltammetric characterization of the electrode, sample solutions were prepared first by adding standard solution of capsaicin into a solution containing 1 part of 96 % ethanol and 3 part glycine buffer at pH 2.5. Cyclic voltammetric measurements were carried out between −0.9 V and +1 V vs. Ag/AgCl with a step potential of 0.01 V and a scan rate of 100 mV s^{-1}.

For pH study, sample solutions at same concentration of capsaicin and at different pH values ranging from pH 2 to pH 7 were prepared by adding standard solution of capsaicin to a 20 mL of solution containing 24 % ethanol and 76 % Britton Robinson buffer. The three electrode system were transferred to a blank buffer solution at each pH value and cyclic voltammograms were recorded at scan rate value of 100 mV s^{-1}. Measurements were carried out like the same way with all the prepared samples at different pH values.

For the repeatability test of the electrodes, 10 measurements of capsaicin were carried out repeatedly on same concentration of capsaicin. To check the stability of the electrode 35 µM capsaicin were measured in a cyclic manner for 14 cycles. Before each sample measurement a blank buffer measurement was taken. Each cycle was followed by a chemical cleaning of the electrode surface which involves rinsing the surface in specific media (48 % ethanol) for 60 s with stirring.

For the real sample analysis, the extracted sample described in the section 2.5 was diluted to 4 times with 0.266 mol L^{-1} glycine buffer so that the final sample solution will have concentration of 0.2 mol L^{-1} glycine buffer containing 24 % ethanol i. e. the same background electrolyte as the previous measurements during this entire capsaicin study. For doing this 25 mL of the extracted sample was transferred to a 100 mL volumetric flash and filled up to the mark with glycine buffer. Cyclic voltammograms of 25 mL of this prepared sample were then recorded. Experimental conditions adopted in this measurement were same as mentioned in section 2.6.

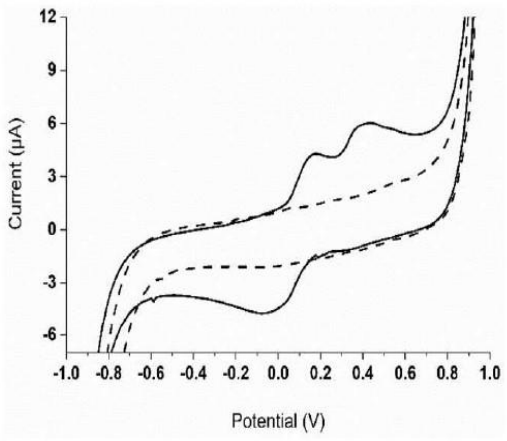


Fig. 2. Cyclic voltammograms obtained from the 2nd scan of background buffer (dashed lines) and 60 μM capsaicin (solid line) prepared in glycine buffer at pH 2.5, using the TiO_2 modified graphite epoxy resin electrode.

3 Results and Discussions

3.1 Voltammetric Behavior of the prepared Electrodes towards Capsaicin

Cyclic voltammogram obtained for 60 μM capsaicin solution by following the analytical procedure described in the section 2.6 is shown in the Figure 2. It is observed that capsaicin gives rise to two well defined oxidation peaks in the potential of +0.17 V and +0.43 V, respectively (this is observed after the 1st voltammertic scan, the one that generates the intermediate). Corresponding reduction peak was observed at −0.05 V. It is observed that the oxidation as well as reduction peaks appear at lower potentials with this TiO_2 modified electrode in comparisons to the previous studies obtained in the literature [36,37,38,40]. A comparison of positions of oxidation peaks of capsaicin obtained from different sensors are added to the suplimentary information, Table S.1. This means then, that oxidation of capsaicin with our developed electrode needs lower energy then usual originated in some catalytic effect.

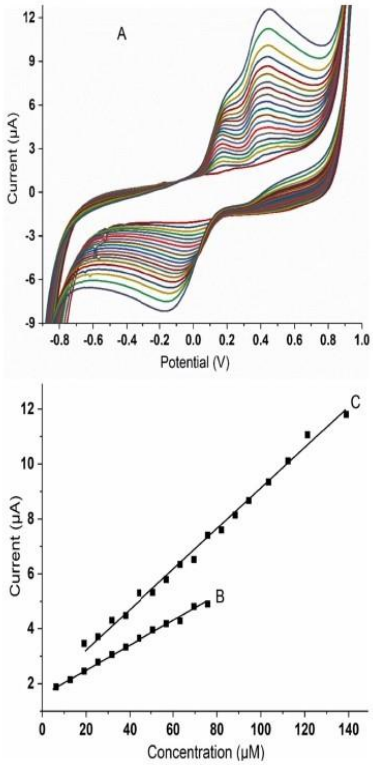


Fig. 4. Cyclic voltammograms in glycine buffer, pH 2.5 at increasing concentration of capsaicin, (6–138 μM) (A); Calibration line of capsaicin for the 1st oxidation peak (+0.17 V), (B); Calibration line of capsaicin for the 2nd oxidation peak (+0.43 V), (C).

3.2 Optimization of Working pH

To obtain the optimum pH for the determination of capsaicin, effect of pH was investigated by measuring of

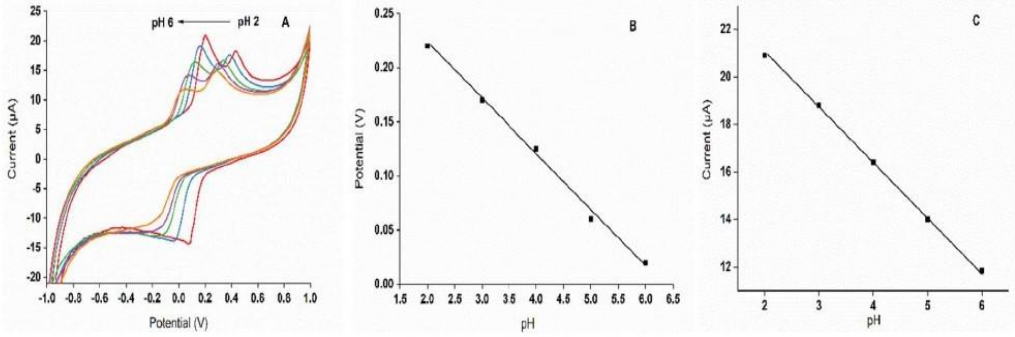


Fig. 3. Current Vs. Potential curve for 40 μM capsaicin at different pH of the Britton Robinson buffer solution (A), Potential of the 1st oxidation peak vs. pH curve (B), Maximum current for the 1st oxidation peak vs. pH curve (C).

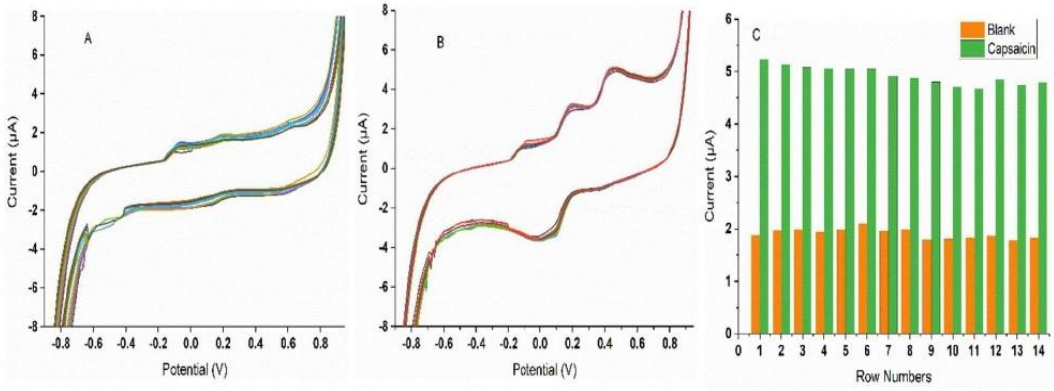


Fig. 5. Current vs. Potential curves for repetitive measurements of blank after each cycle (A), repetitive measurements of 35 μM capsaicin in a buffer/capsaicin cycle manner (B), comparisons of blank and capsaicin current measurements at +0.43 V (C).

Table 1. Comparison of analytical properties of different electrodes in the literature used for the voltammetric determination of capsaicin.

Working electrode	Electro-chemical technique[a]	pH media	Preconcen-tration time	LOD (μM)	Linearity range μmol L^{-1}	(RSD) %	Ref
Multi walled carbon nanotubes based screen printed electrode	CV	1.0	60 s	0.45	0.5–35	–	36
Boron doped diamond electrode	SW	1.0	90 s	0.039	0.16–20	4.44	37
Amino-functionalized mesoporosus silica	LSV	3.0	180 s	0.02	0.040–0.4, 0.4–4	–	38
Graphite pencil electrodes	ASV	9.0	120 s	0.0037	0.016–0.32	7.1	45
Mesoporous cellular foams	DPV	1.0	60 s	0.08	0.76–11.65	–	39
Ag/Ag_2O poly (sodium4-styrensulfonate) reduced graphene	DPV	1	60 s	0.4	1–60	9.70[b]	49
Graphene-titania-nafion	LSV	1	10 min	0.0086	0.03–10	–	42
Carbon nanotubes and ruthenium nanoparticles	SW	4	–	0.0025	0.01–0.4	5[c]	43
Graphite pencil electrodes	CV	9	–	0.1	0.1–100	–	46
Carbon paste electrodes modified by β-cyclodextrin	CV	1 mol L^{-1} $HClO_4$	–	0.065	1.44–33	–	40
Graphite epoxy resin electrode with TiO_2 nanoparticles	CV	2.5	–	5.34	5.34–138	2.53	this work

[a] Cyclic voltammetry (CV), linear sweep voltammetry (LSV), adsorptive stripping voltammetry (ASV), differential pulse voltammetry (DPV), square wave voltammetry (SW), [b] peak current decreased by 9.70 % after five hundred times determination. [c] peak current changes by less than 5 % after measuring two weeks

Table 2. Results of calculated concentrations of capsaicin in different real samples.

Pepper samples	Amount of capsaicin determined (μM)	Confidence level (95 %)	Actual amount of capsaicin
Delhuerto hot pepper sauce	15.59	±1.49	–
Tabasco habanero pepper sauce	24.68	±2.12	–
Alacapsin	29.06	±2.44	30.7

40 μM of capsaicin. Voltammograms obtained from the 2nd scan of 40 μM capsaicin solution prepared in different pH solutions starting from pH 2 to 6 are shown in the Figure 3(A). Figures 3(B) and 3(C) show plots of potential vs. pH value and peak current corresponding to 1st oxidation peak vs. pH. It is observed from 3(B) that the peak potential of oxidation peak of capsaicin varies linearly with the pH and shifts to the negative potential

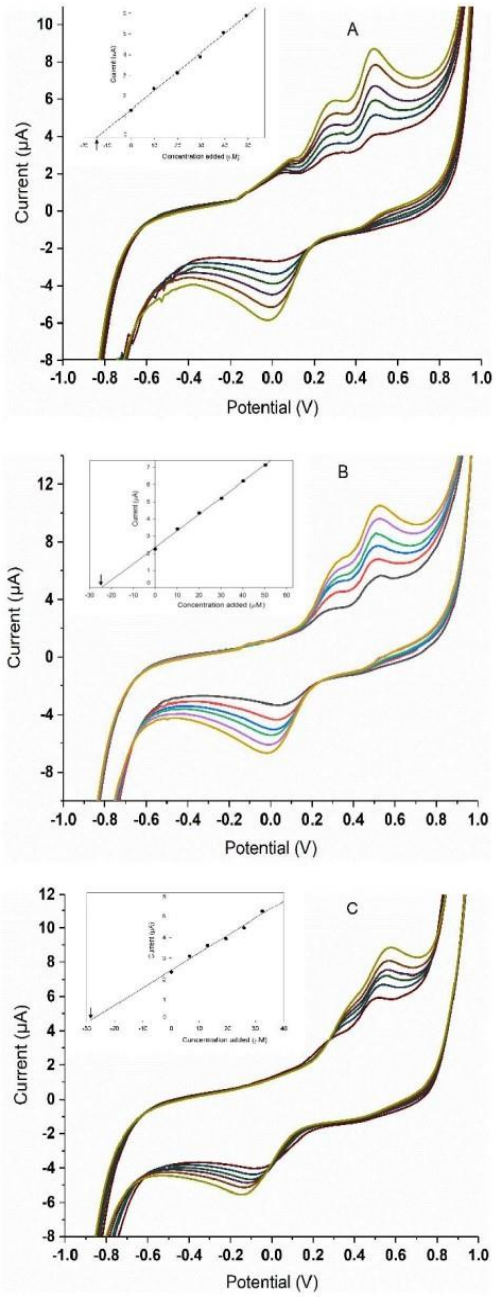


Fig. 6. Cyclic voltammetric signals for additions of 10, 20, 30, 40 and 50 μM capsaicin on extracted Delhuerto hot pepper sauce (A); 10, 20, 30, 40 and 50 μM capsaicin on extracted Tabasco habanero pepper sauce (B); 6.5, 13, 19.5,26, and 32.5 μM capsaicin on the extracted Alacapsin. (C); All the measurements were carried out in glycine buffer solution (25 % EtOH) pH 2.5 at open circuit condition, $\upsilon = 100\ mV\,s^{-1}$. The first scans show the signal of diluted real sample before the additions of capsaicin.

by 51 mV per unit increase in the pH with a regression equation ($Ep = -0.051pH + 0.323$, $R^2 = 99$). This implies that protons participate in the electrochemical process. The calculated value of the slope is close the the theoretical value of $59\ mV\,pH^{-1}$. This suggests that equal amount of protons and electrons are exchanged during the oxidation reaction of capsaicin. From the Figure 3 (C) it is observed that the oxidation peak current decreases linearly with the increase of the pH value from pH 2 to pH 6. So to avoid extreme acidity on electrode surface a pH of 2.5 (accomplishable with glycine buffer) was selected for the subsequent experiments.

3.3 Characterization of Analytical Properties

To check the analytical performance of the electrode, a calibration experiment was carried out in $0.2\ mol\,L^{-1}$ glycine buffer with $0.1\ mol\,L^{-1}$ KCl saline background. Figure 4 shows the voltammograms obtained upon additions of capsaicin. From the two oxidation peak, two calibration curves were obtained by plotting peak current vs. added concentration of capsaicin. Calibration line on Figure 4(B), obtained from the oxidation peak at +0.17 V is linear within the concentration range 6 to 75 μM with a regression equation $I(\mu A) = 0.044(\pm 0.0021)\cdot C(\mu M) + 1.63$ (± 0.11) having correlation coefficient 0.993 ($n = 12$). Calibration line on Figure 4(C), obtained from the oxidation peak at +0.43 V is linear within the concentration range 12 to 138 μM with a regression equation $I(\mu A) = 0.075(\pm 0.0037)\cdot C(\mu M) + 1.64(\pm 0.31)$ having correlation coefficient 0.996 ($n = 17$). Limit of detection values were calculated as 5.34 and 11.3 μM for the oxidation peak at +0.17v and at +0.43 respectively (using the formula of $3.3S_{y/x}/m$).

To check the repeatability of our developed electrode, ten measurements of 23.33 μM capsaicin in glycine buffer were carried out in series by following the experimental conditions described in the section 2.6. The relative standard deviation of responses was obtained as 2.53 % which indicates a good repeatability of the electrode responses towards capsaicin. From the stability study of capsaicin as explained in the analytical procedure in section 2.6, RSD values of 3.41 % and 2.31 % were obtained for the capsaicin and blank buffer solutions, respectively. When this stability experiment was reproduced with the standard graphite epoxy resin electrode without any modification, RSD values obtained for capsaicin and blank solutions were 14.29 % and 9.46 %, respectively, even considering the electrode surface was rinsed with ethanol/water solution after each cycle. These values illustrate the significant fouling effect associated to the capsaicin determination, as already commented. Figure 5 illustrates the results obtained in the stability study, when the TiO_2 modified electrode was used, where a correct recovery of the response can be observed. To the best of our knowledge this attempt of checking fouling carried out by capsaicin is reported for the first time, and demonstrates how important this issue can be when not

using disposable electrodes. With our TiO_2 modified electrode and with a simple rinsing step, electrochemical determination of capsaicin results in a very stable series of blank measurements, indicating there is no memory effect, and with a controlled variability for the series as capsaicin standard measurements, demonstrating the minimization of the fouling effect. Furthermore reproducibility of polishing was also carried out for both GEC and TiO_2 modified electrodes and RSD values were found to be as 3.19 % and 3.27 % respectively for capsaicin which implies that our developed sensor have similar RSD values for both polishing and cleaning with ethanol and water mixture whereas GEC does not have it. Hence our sensor can be used for capsaicin determination by cleaning only instead of using time consuming polishing procedure. A scenario of comparison of our developed sensor with another previously developed sensors found in the literature is shown in Table 1, where it is observed that the value for repeatability of measurements (RSD) is lowest for our developed sensor. Although LOD is not among the best in the table, it is to be commented our procedure is by direct voltammetry while best sensitivities are obtained in stripping conditions.

3.4 Analysis of Real Sample

To show final application of the developed sensor towards real samples containing capsaicin, measurements were first carried out in the diluted samples of extracted solutions of capsaicin as explained in the section 2.6. After measuring the diluted samples, to obtain an accurate concentration of capsaicin in the real samples, standard additions of capsaicin were performed in each sample and voltammograms were recorded. It is observed that the voltammograms obtained from this extracted samples of capsaicin are not exactly similar with respect to shape and size to that had been obtained for the pure capsaicin samples shown in the Figure 1. This matrix effect may be because of interfering agents present in the real sample. This problem is overcome by doing baseline corrections to the voltammograms obtained from the real samples using OriginPro 2017 software after measuring. Additionally, estimation of capsaicin was carried out by using the peak at the second oxidation potential (+0.43 V) as the obtained voltammograms are clearer to interpret. Figure 6 and their insets depicts the voltammograms obtained in the real sample Delhuerto hot pepper sauce (A),Tabasco hot pepper sauce (B) and Alacapsin (C) and the corresponding standard addition curve, respectively. Average of concentration results obtained from 3 replicates of each real sample is given in the Table 2

4 Conclusions

The developed capsaicin electrochemical sensor has shown interesting response features versus capsaicin and can be a good alternative to human-subjective Scoville organoleptic test; it can also be further developed as a portable electrochemical sensor for the estimation of capsaicin content in food and pharmaceutical products. In addition to this, to the best of our knowledge, our proposed sensor employing titanium nanoparticles is the first sensor to be developed which show very reduced fouling effect with capsaicin, this is accomplished through the use of chemical cleaning of the electrode surface which involves rinsing the surface in specific media (48 % ethanol). The developed sensor demonstrated that can be applied in the repeated measurements of capsaicin samples without renewing the electrode surface between consecutive measurements.

Acknowledgements

Financial support for this work was provided by Spanish Ministry of Economy and Innovation, MINECO (Madrid) through projects CTQ2016-80170-P. M. S. thanks to AGAUR, Generalitat de Catalunya and to European Social Fund, European Union for FI fellowship. M. d. V. thanks the support from program ICREA Academia.

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Received: June 27, 2019
Accepted: September 25, 2019
Published online on October 22, 2019

6.2. Publication 2:

Optimization of Sensors to be used in a Voltammetric Electronic Tongue Based on Clustering Metrics

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Sensors, 2020, 20, 4798.

sensors

MDPI

Article

Optimization of Sensors to be Used in a Voltammetric Electronic Tongue Based on Clustering Metrics

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Received: 31 July 2020; Accepted: 21 August 2020; Published: 25 August 2020

Abstract: Herein we investigate the usage of principal component analysis (PCA) and canonical variate analysis (CVA), in combination with the F factor clustering metric, for the a priori tailored selection of the optimal sensor array for a given electronic tongue (ET) application. The former allows us to visually compare the performance of the different sensors, while the latter allows us to numerically assess the impact that the inclusion/removal of the different sensors has on the discrimination ability of the ET. The proposed methodology is based on the measurement of a pure stock solution of each of the compounds under study, and the posterior analysis by PCA/CVA with stepwise iterative removal of the sensors that demote the clustering when retained as part of the array. To illustrate and assess the potential of such an approach, the quantification of paracetamol, ascorbic acid, and uric acid mixtures were chosen as the study case. Initially, an array of eight different electrodes was considered, from which an optimal array of four sensors was derived to build the quantitative ANN model. Finally, the performance of the optimized ET was benchmarked against the results previously reported for the analysis of the same mixtures, showing improved performance.

Keywords: electronic tongue; voltammetric sensors; principal component analysis; artificial neural networks; discrete wavelet transform

1. Introduction

Electronic tongues (ETs) are analytical systems based on the combination of an array of sensors with low-selectivity and/or cross-response features in order to obtain some added value in the generation of analytical information. These are coupled with advanced chemometric tools that allow the interpretation and extraction of meaningful data from the complex readings [1,2]. Thus, the selection of the sensor array that will comprise the ET is a key step that will highly influence the performance of the system [3].

Despite its importance, most of the papers dealing with ETs focus on the developed application itself or the data treatment stage, but very few report on the choice of the sensors. The challenge here arises on the a priori selection of the best combination of sensors that can carry out the desired qualitative or quantitative task given the difficulty to assess the cross-reactivity shown between them and the impact that this will have in the final model. In this direction, one common approach that has been taken in the case of potentiometric sensors is the inclusion of at least one or two ion-selective sensors (ISEs) towards the compounds of interest plus some generic ones; however, this does not guarantee that the optimal array is selected [1]. Another approach would be the complete sensor characterization, which consists in carrying out the multi-analyte calibration, from which the surface response plots are built and the binary selectivity coefficients are calculated (e.g., Reference [4]). However, this is a tedious task even for the two-analyte case, and it does become not feasible for more

complex mixtures as it may require hundreds of samples. Furthermore, such a task becomes even more complex when, e.g., voltammetric sensors are used instead of potentiometric ones due to the higher complexity and dimensionality of its response. Alternatively, another approach that has been employed is the use of feature selection methods upon measurement of all the samples, and to carry out the a posteriori removal of the variables and/or sensors that do contribute less to the classification or quantification task (e.g., usage of genetic algorithms or other pruning methods [5]). Although this approach does probably provide the best outcome possible, it is also more tedious and requires instruments capable of simultaneously measuring a large number of channels.

In the same direction, we had also reported previously on the usage of the autocorrelation between the signals of the different sensors that form the sensor array as an objective criterion for the selection or removal of redundant sensors in voltammetric arrays [6,7]. Although it provides a measure of sensors' response redundancy, it is purely based on the signal, but not on the cross-reactivity towards the analytes of interest. More recently, principal component analysis (PCA) taking the transposed data matrix has also been suggested as a guiding method to select the best sensing units to compose the array of an ET [8]. However, the authors themselves conclude that further experiments are required to confirm the potential of the method.

Therefore, the development of a simple methodology that allows the a priori selection of the optimal sensor array to carry out a specific application is of utmost interest. In this direction, herein we propose the usage of PCA in combination with some clustering metrics as a tool to carry out such selection. The former allows us to visually compare the performance of the different sensors, while the latter allows us to numerically assess the impact that the inclusion/removal of the different sensors does have in the discrimination ability of the ET towards the compounds of interest.

In order to demonstrate and illustrate such a procedure, the simultaneous quantification of paracetamol (PA), ascorbic acid (AA), and uric acid (UA) mixtures was chosen as the study case. These mixtures correspond to a common case in the pharmaceutical field where the determination of paracetamol in the presence of ascorbic acid is attempted. The latter is usually present as an excipient, whereas the inclusion of uric acid is motivated as some studies suggest that ascorbic acid intake is related to uric acid concentration in serum [9,10]. This particular case was chosen as this mixture has already been previously analyzed in our laboratories employing different sensors arrays, thus providing us with guidance on which performance could be expected and whether previous results could be improved or not by tailoring the electrode choice to particular cases.

In this direction, the present work aims to demonstrate the advantages derived from the tailored selection of the sensor array for each ET developed application. Upon measurement of stock solutions of the different active pharmaceutical ingredients (APIs), those were transformed by using PCA/CVA, which in combination with the F factor metric allowed the selection of the different sensors. Next, a quantitative model was built by means of artificial neural networks (ANNs) to achieve the simultaneous determination of the three APIs, the performance of which was benchmarked against previously reported ETs.

2. Materials and Methods

2.1. Reagents and Apparatus

All reagents were of analytical reagent grade and were used as received without any further purification. All the buffer solutions were prepared in ultrapure water (18.2 MΩ·cm) purified by a MilliQ System (Millipore, Billerica, MA, USA). Potassium hydrogenphosphate, potassium dihydrogenphosphate and sodium chloride, which were used for the preparation of the phosphate buffer, were purchased from Merck (Darmstadt, Germany).

The active pharmaceutical ingredients (APIs), paracetamol and uric acid, were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas ascorbic acid was purchased from Panreac Química SLU (Barcelona, Spain). For the modification and preparation of the electrodes, we used nanoparticles of

bismuth (III) oxide, titanium (IV) oxide, zinc (II) oxide and tin (IV) oxide, plus cobalt (II) phthalocyanine and polypyrrole purchased from Sigma-Aldrich (St. Louis, MO, USA), while Prussian blue was obtained from Acros Organics (Geel, Belgium). Graphite powder (particle size < 50 μm) used for the construction of the electrodes was purchased from BDH Laboratory Supplies (Poole, UK), and Epotek H77 resin and the corresponding hardener were obtained from Epoxy Technologies (Billerica, MA, USA).

All the electrochemical measurements were carried out in a PGSTAT 30 Autolab potentiostat (EcoChemie, The Netherlands) with GPES 4.7 version software (EcoChemie). Voltammetric measurements were conducted using a conventional three-electrode cell configuration where a combined electrode (Crison 5261, Barcelona, Spain), made up of a metallic platinum wire and an Ag/AgCl electrode was used as both the auxiliary and reference electrode.

2.2. Sensor Array

An array of seven different modified graphite epoxy composite (GEC) electrodes was initially prepared to be evaluated as the working electrodes that will form the ET [11]. Briefly, for the construction of the working electrodes, first of all, a shaped copper disc was soldered to an electrical connector, and then introduced into a 6 mm internal diameter PVC tube, resulting in a cylindrical cavity. A paste was then made by mixing 15% of graphite powder, 2% of the specific modifier, and the epoxy resin and the hardener (in the ratio 20:3 w/w). Next, this paste is loaded into the cavity of the PVC tube and cured at 80 °C for 3 days. Afterwards, using emery papers of decreasing grain size, electrode surfaces were polished until a flat shiny surface appeared. One of the main advantages of such electrodes is that re-polishing of the surface using emery paper allows the regeneration of the electrode, recovering any loss of their response.

In this manner, seven different GEC electrodes, each of them modified with cobalt (II) phthalocyanine (CoPc), polypyrrole (PPy), Prussian blue (PB), oxide nanoparticles of bismuth (Bi_2O_3), titanium (TiO_2), zinc (ZnO) and tin (SnO_2), were prepared. Additionally, a Pt disc electrode was also prepared and used to complete the eight-sensor array. The Pt disc electrode was constructed by soldering a Pt wire (99.95% purity, 1 mm diameter) to an electrical connector and then introducing the connector into a PVC tube. The wire was then coated in epoxy resin (exposing only the wire cross-section) and cured at 80 °C for 3 days.

Such modifiers were selected taking into account previous studies with ETs. Prussian blue is well-known to be an electron mediator in the development of many biosensors, which has also been used in the development of ETs [12–14]. The usage of nanoparticles has emerged as an alternative to the respective bulk metals given its higher surface/mass ratio and improved electrochemical properties [13,15,16]. Similarly, conducting polymers such as polypyrrole have electrocatalytic and antifouling properties [17–19], while phthalocyanines are reported to be efficient electrocatalysts in the determination of many important inorganic, organic, or biological compounds [20,21]. Lastly, the use of bare metal electrodes corresponds to one of the more common choices for the development of voltammetric ETs [22–24].

2.3. Samples Preparation and Voltammetric Measurements

All APIs stock solutions were prepared in 0.05 M phosphate buffer at pH 7.0 with 0.1 M KCl as saline background/supporting electrolyte. For the optimization of the sensor array, 250 μM stock solution of each of the APIs were measured separately in order to investigate their electrochemical behavior with the different sensors. Upon selection of the optimal sensor array, calibration curves for the APIs were built and their analytical response was further characterized in terms of linearity, sensitivity, limit of detection (LOD), reproducibility, etc. To this aim, solutions of increasing concentration of each API were prepared from the stock solutions in phosphate buffer and measured under the below conditions.

For the simultaneous analysis of APIs mixtures, a set of samples consisting of mixtures of the three compounds were prepared. Samples were divided into two subsets: the training subset based on

a tilted 3^3 factorial design (27 samples), which was used to build the quantitative model [25], and the testing subset with samples randomly distributed along the experimental domain (11 samples) that was used to assess the actual performance of the built models.

The electrochemical behavior of all the APIs and their mixtures was assessed by recording a complete cyclic voltammogram between −0.7 V and +1.2 V vs. Ag/AgCl with a step potential of 10 mV and a scan rate of 100 $mV \cdot s^{-1}$, without the application of any pre-conditioning potential or accumulation time. Furthermore, to avoid any fouling effect or drifts during the measurements, a blank measurement in phosphate buffer was carried out after each measurement.

These conditions were used for all the experiments, except during initial experiments for the selection of the sensor array, that in order to ensure that the potential window was wide enough to see any possible peak, voltammetric measurements were carried out in the range −1.5 V to +1.5 V, but keeping all the other conditions unaltered. Similarly, as a 6-channel multipotentiostat was employed, final measurements were carried simultaneously, but some of the initial measurements for sensor selection had to be carried sequentially in groups.

2.4. Chemometric Analysis

Data analysis was carried out in Matlab 7.1 (MathWorks, Natick, MA, USA) by specific routines developed by the authors using the Statistics, Wavelet, and Neural network toolboxes [26–28]. Final representation and analysis of the data were done with the aid of Sigmaplot (Systat Software Inc., San Jose, CA, USA).

Recorded voltammograms were first compressed with discrete wavelet transform (DWT), which allowed us to decrease the dimensionality of the data while preserving the relevant information [26]. Next, PCA and canonical variate analysis (CVA) were used for the qualitative analysis of the data and a tailored selection of the ET array. Finally, ANNs were used to build the simultaneous quantitation models of the ternary mixtures.

For the selection of the optimal sensor array, data was submitted to PCA and CVA, and the clustering observed was evaluated by means of the F factor [29–31]. The F factor is defined as the ratio of variances between different clusters and the sum of internal variance in all clusters (Equation (1)). It can be used as a standard procedure to measure the capability of a particular sensor to discriminate between different classes of samples in ET applications. Since the F factor compares the variability between classes to variation within classes, with increasing F values the discrimination between different classes becomes easier.

$$F = \frac{\frac{\sum_{i-1}^{k} n_i \left(\overline{z_i} - \overline{\overline{z}}\right)^2}{k-1}}{\frac{\sum_{i-1}^{k} \sum_{j-1}^{n_i} \left(z_{ji} - \overline{z_i}\right)^2}{\sum_{i-1}^{k} n_i - k}} \quad (1)$$

where k is the number of classes, i the number of following class, j the following number of the sample in i-th class, n_i the number of samples in i-th class, and z_{ji} the sensor response for j-th sample in i-th class, and $\overline{z_i}$ and $\overline{\overline{z}}$ are the mean value of a sensor response in a particular class of samples and the mean value of sensor response for all samples, respectively. These can be defined as:

$$\overline{z_i} = \frac{\sum_{j=1}^{n_i} z_{ji}}{n_i} \quad (2)$$

$$\overline{\overline{z}} = \frac{\sum_{i=1}^{k} \sum_{j=1}^{n_i} z_{ji}}{\sum_{i=1}^{k} n_i} \quad (3)$$

3. Results and Discussion

3.1. Selection of Sensors

The aim of this work is to demonstrate the advantages derived from the tailored selection of the optimal sensor array for each case when developing ET applications, and to assess whether this can be done a priori taking only single measurements of the stock of each of the compounds that we aim to analyze (Figure 1).

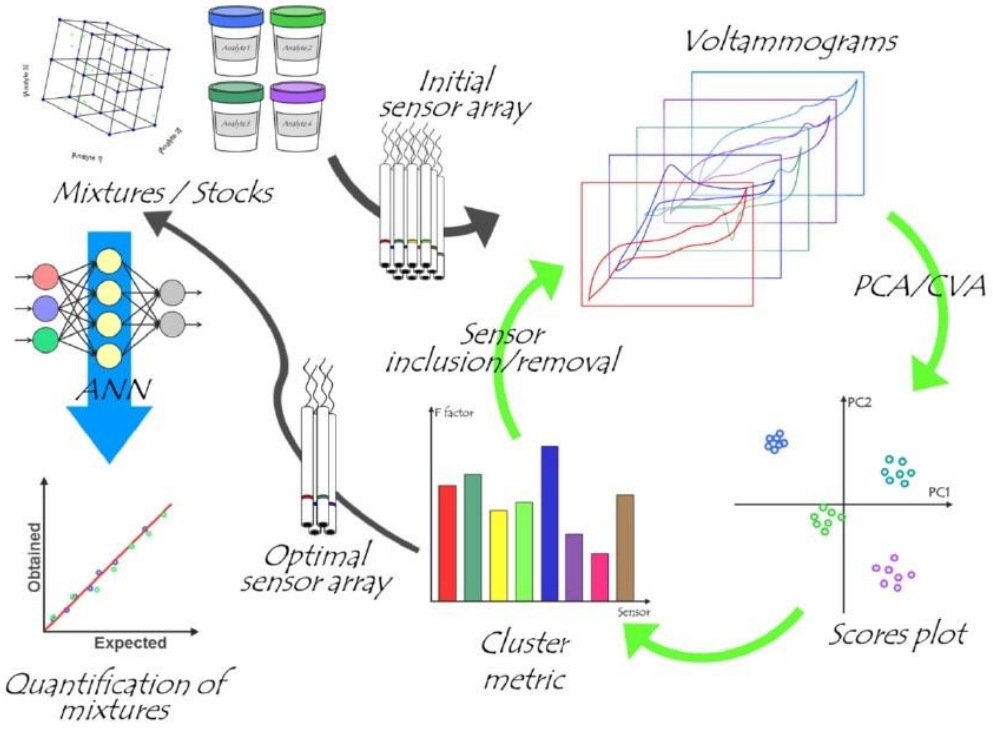

Figure 1. Schematic representation of the methodology followed for the a priori selection of the optimal sensor array. Briefly, stock solutions of each of the analytes are measured with all the considered sensors, obtaining a voltammogram for each of them. Next, those are submitted to PCA/CVA, and the clustering is evaluated by means of the F factor. This is repeated, leaving out of the analysis each of the sensors of the array (one at a time), and the one that leads to the higher improvement is removed. The whole process is repeated until a decrease in the F factor is observed after discarding one of the sensors. Finally, with the selected sensor array, the quantitative application is carried out.

To this aim, we took as a study case the analysis of three different APIs (PA, AA and UA), and attempted to choose the most appropriate electrodes for their electrochemical analysis. When choosing the optimal electrodes, it has to be kept in mind how important the cross-responses of the different electrodes towards the analytes of interest and among them are in ET applications. That is, each electrode shows a differentiated response between each of the analytes, and the different sensors show a differentiated response between them, so that they jointly allow us to differentiate the different compounds [3].

In this direction, the first step was to assess the voltammetric responses of each of the electrodes towards the individual compounds. For doing so, five replicate samples of the 250 µM solutions of each of the APIs were prepared, and the voltammetric measurements were carried as described in Section 2.3. An extract of the responses obtained for the different electrodes is shown in Figure 2. As it

can be seen, the voltammetric profiles of each of the sensors are found to be different, with all of them showing distinct responses for each of the compounds.

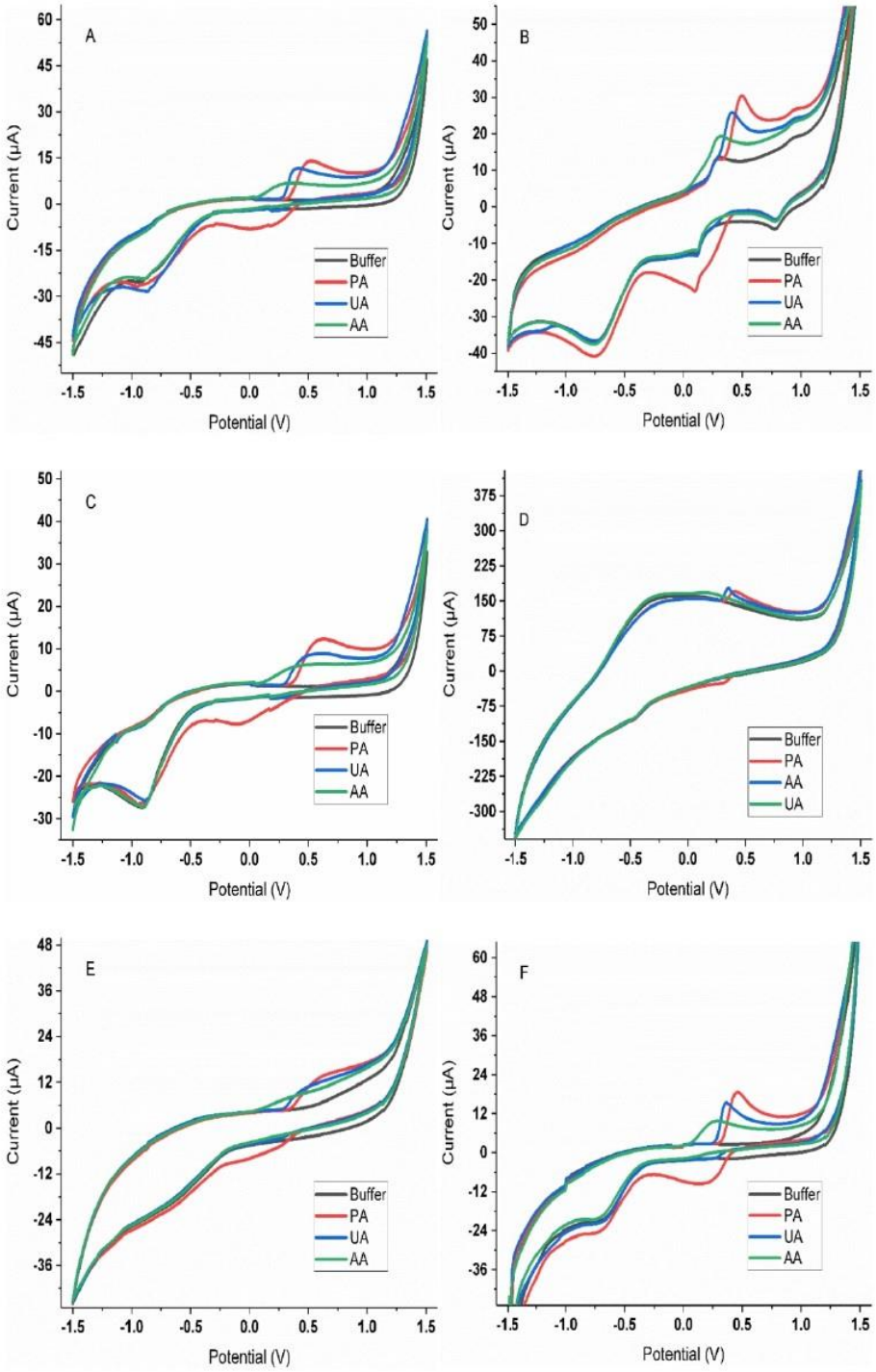

Figure 2. *Cont.*

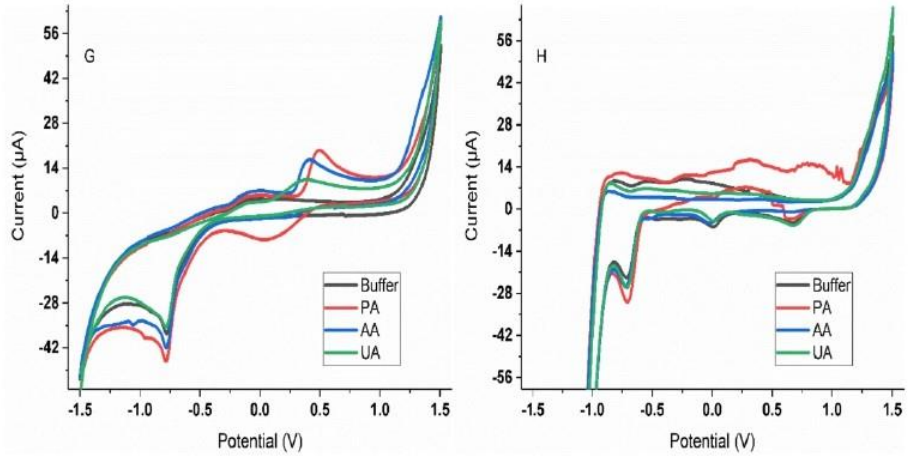

Figure 2. Voltammograms obtained for the three APIs (250 μM in phosphate buffer) using the GECs modified with (**A**) SnO_2, (**B**) Prussian Blue, (**C**) ZnO, (**D**) PPy, (**E**) CoPc, (**F**) TiO_2 and (**G**) Bi_2O_3, and (**H**) the metallic Pt electrode.

To objectively carry out the selection of the final sensor array for the quantitative task, the next step was the compression of the data with DWT and its analysis by means of PCA and CVA. Firstly, the 2D score plots were obtained, and from those the F factor was calculated. This process was done with the 8-sensor array and repeated by removing one sensor at a time, similar to what could be considered a leave-one-out process, but from the sensors' side (Figure 1). Next, the F values for each of the iterations were compared, and the sensor that when excluded led to the higher increase of the F index was then discarded. This process was repeated, reducing one-by-one the selected sensors, until the F value decayed (Figure 3).

In this manner, from the eight sensors initially considered, the first one that was discarded was the one modified with SnO_2 nanoparticles, which led to a significant improvement in the clustering (the F value increased from 4.19 to 5.19). As stated, this process was repeated, taking the remaining seven sensors as reference for the F value, and leaving out again one sensor at a time for the calculations; the Bi_2O_3 nanoparticle modified sensor was discarded this time. In the next iteration, TiO_2 modifier was discarded as no significant increase/decrease in the F value was observed. Thus, it was considered that its inclusion/exclusion does not demote the performance of the system. Lastly, the cobalt(II) phtalocyanine modified sensor was also discarded, whereas in the next iteration no further sensors were removed, as even the exclusion of the one with a larger F value implied a decrease of this parameter. In this manner, the selected sensor array was formed by four electrodes: a metallic Pt sensor and GECs modified with ZnO nanoparticles, Prussian blue, and polypyrrole, and the maximum F value achieved was 5.75.

The resulting PCA score plot obtained with the reduced sensor array is shown in Figure 4B, with an accumulated explained variance of ca. 82.3 %; a large value that reflects how most of the variance contained in the original data is now summarized with only these two coordinates (PCs). More importantly, we can observe how clear clusters are obtained for each of the compounds and how easily these can be distinguished from each other. Hence, based on the voltammetric profiles and the PCA score plot, we can say that the initial selection of sensors based on the F factor calculation seems to be a satisfactory step towards the electrochemical quantification of the three APIs. Figure 4A, on the other hand, shows the departing point with the eight-sensors array, and where the clustering is just preliminarily worked out. As an additional comment, it is also evident how the optimization of the array is also able to amend the drift content incorporated in the original set of sensors.

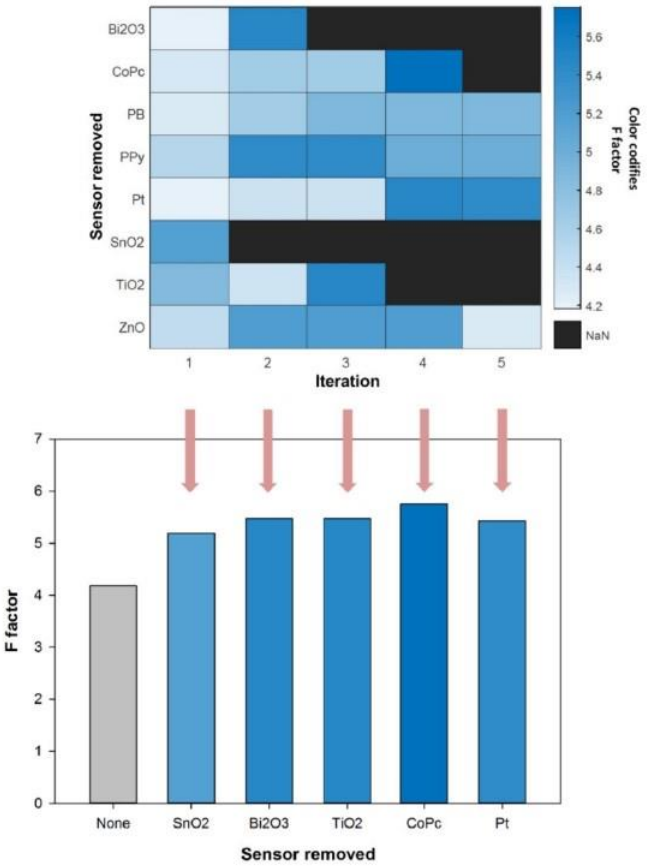

Figure 3. (Top) Color map of the variation of the F values after iterative exclusion of the different electrodes; the Y-axis shows the sensor being left out for the calculation of the F factor and the X-axis shows the successive iterations for the selection of the less significant sensor. (Bottom) Bar plot of the changes of the F values after exclusion of the sensor that leads to the biggest F value at each iteration. In both cases, the color of the plot codifies the F factor values as per the color bar. Iteration 5 does not produce any further improvement, and then the process is stopped.

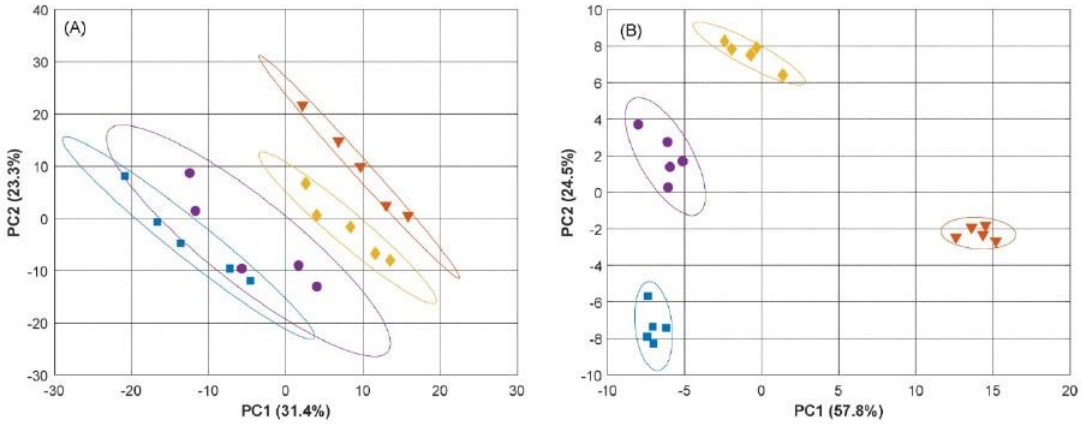

Figure 4. Score plot obtained from the DWT-PCA of five replicas of individual APIs using (**A**) the eight-sensor array or (**B**) the selected four-sensor array. (■) Buffer, (▼) paracetamol, (●) ascorbic acid and (♦) uric acid. Ellipses plotted correspond to 95% confidence limits for each of the clusters.

Lastly, the analysis of the loadings biplot is not very significant in this case as what we aim to evaluate is not only which features are more relevant, but which improve more the discrimination capabilities of the ET. However, it is quite well known that since PCA is focusing only on the data variance, external factors, e.g., drift on the sensors response, can dominate it. Therefore, such information cannot be only obtained from the scores and loadings plot, but from the F factor. Moreover, the large number of variables registered when voltammetric sensors are used (hundreds for each of the sensors) makes the interpretation of the loadings plot difficult/cumbersome. However, a numerical inspection of the loadings contribution from the magnitude of its vector allowed us to confirm that in both cases all the sensors contribute significantly to the obtained scores plot, with most of the loadings very close to the correlation circle: ca. 81% of them with a magnitude as big as 0.6 times the one of the loading with the highest contribution for (A), and 72% for (B). In terms of sensors, the number of variables for each of them that contribute to those percentages are 15 to 30% for (B), and 2.2 to 17% for (A) (data not shown).

3.2. Characterization of the Analytical Response

Upon selection of the reduced sensor array, the next step was the characterization of the voltammetric responses of each of the electrodes towards the individual APIs. On the one side, we wanted to confirm that, actually, different sensitivity was shown by the different sensors, as well as assess its linear range to restrict the experimental domain for the quantitative experiment. On the other side, we wanted to also assess sensors' repeatability, as this is a critical parameter when working with ETs, which require performing a large number of consecutive measurements.

3.2.1. Calibration Curves

Prior to building the quantitative model for the mixtures' analysis, the linear range and sensitivity of the different electrodes were evaluated. Individual calibration curves were built for each of the selected four sensors towards each of the APIs. To do so, stocks of increasing concentration within 0 to 500 µM for paracetamol and uric acid, and 0 to 2000 µM for ascorbic acid were prepared and measured as described in Section 2.3. From the recorded voltammograms, the maximum peak height was taken and plotted against its concentration (data not shown). From those, the fitted regression equations are summarized in Table 1. As represented by the large value of the coefficient of determination (R^2), it can be concluded that good linearity was obtained within the above ranges for all the APIs and sensors. Hence, those same ranges were selected for the final quantitative model. Moreover, we can confirm how different sensitivities (calibration slopes) are obtained for each of the sensors and compounds, a situation that we aimed for with the selection of the sensor array (i.e., to avoid having redundant sensors or sensors that do not contribute to the discrimination of the different compounds).

Table 1. Calibration data (y vs. x) for the individual calibrations of paracetamol, ascorbic acid, and uric acid employing the final sensors in the array.

Electrode modifier		ZnO	Prussian Blue	Polypyrrole	Metallic Pt
Paracetamol	Equation	y = 0.0598x + 0.613	y = 0.0629x + 10.2	y = 0.0857x + 29.4	y = 0.0027x + 1.08
	R^2	0.9971	0.9993	0.9989	0.9589
	LOD[1] (µM)	28.4	13.9	17.2	59.4
Ascorbic acid	Equation	y = 0.0152x + 0.989	y = 0.0261x + 6.93	y = 0.0304x + 21.0	y = 0.0015x + 0.634
	R^2	0.9977	0.9990	0.9987	0.9624
	LOD[1] (µM)	59.3	68.5	77.5	417
Uric acid	Equation	y = 0.0452x + 1.12	y = 0.0499x + 10.8	y = 0.0668x + 31.0	y = 0.0028x + 1.02
	R^2	0.9988	0.9983	0.9995	0.9967
	LOD[1] (µM)	17.9	23.5	9.35	28.3

[1] Calculated from three times the standard error of the regression.

3.2.2. Stability Measurements

Similarly, since building the quantitative model requires a considerable number of consecutive measurements with the sensor array, it is important to assess whether the repeatability within consecutive measurements for all the sensors is good enough. In this direction, a sample containing 100 μM of each of the APIs was prepared, and the performance of the sensor was evaluated by calculating the relative standard deviation (RSD) obtained after 18 consecutive measurements without changing the sample, equipment, and analyst.

Before the sample measurement, a blank buffer measurement was taken, repeating this cycle up to 18 times. In this manner, we could ensure that no loss of signal was obtained from the measurement of the sample mixture, while the measurement of the blank allowed us to ensure that no drifts on the baseline were being observed. After each cycle, cleaning of the electrode surfaces was carried out in phosphate buffer by recording a cyclic voltammogram under the same conditions. RSD% values of all the sensors were found to be below 4%, indicating good stability of the electrode responses. More specifically, the repeatability expressed as the percentage of RSD over 18 consecutive measurements of blank/standard cycles for the different electrodes forming the final array were: 2.4% for ZnO, 1.99% for PB, 2.4% for PPy and 3.6% for the Pt sensor.

3.3. Quantitative Analysis of APIs Mixtures

Upon selection of the optimal sensor array for the analysis of paracetamol, ascorbic acid, and uric acid, the next step was to evaluate the performance of such an array to achieve the simultaneous determination of their mixtures. To this aim, the set of samples described in Section 2.3 were measured under the same conditions as in previous experiments, recording a complete cyclic voltammogram for each of the electrodes (Figure 5). As could be expected from Figure 2, although a different voltammetric response is obtained for each of the considered compounds individually, there is a clear overlap when mixtures of those are analyzed simultaneously. Therefore, not being possible to achieve its quantification via univariate regression, requiring the aid of chemometric models; an approach that is possible thanks to the cross-response shown by the selected sensors, i.e., the different sensitivity shown by each of the electrodes towards each of the compounds (Table 1). This is a desirable condition for the proper performance of any multisensory array analysis system.

However, before building the quantification model, and especially if ANNs are to be used [32], a preprocessing step to reduce the high dimensionality of the data is required. The benefits derived from such a step are particularly critical with ANNs, as this contributes to preventing the under-determination problem encountered with an oversized ANN with excessively complex data, while it also reduces significantly the time and memory required for its modelling. Moreover, in general, it also leads to models with better performance and generalization ability as it avoids redundancy in the input data and reduces their complexity with the risk of overfitting [26]. In our case, this compression was achieved by means of DWT, using the Daubechies wavelet mother function and a fourth decomposition level. This allowed us to reduce the initial 1696 data points (currents corresponding to 424 polarization potentials × 4 sensors) down to 132 coefficients, representing a reduction of 92.2% without any loss of relevant information ($r > 0.99$ and $fc > 0.95$ in the compressed vs. original signal comparison).

For the selection of the neural network topology, a systematic study was carried out in which the number of neurons in the hidden layer, as well as the transfer functions between the input and hidden layer and between the hidden and output layers, were varied. The different ANN models were built employing the data of the training subset, and the selection of the optimal one was chosen from the comparison of the performance towards the testing subset. This data division helped to ensure that more unbiased data was obtained and to detect possible models that were being over-fitted, and consequently to better assess the accuracy of the model. The final ANN architecture had 132 neurons in the input layer (corresponding to the DWT coeffs., 33 × 4 sensors), 6 neurons and purelin transfer function in the hidden layer, and 3 neurons (one for each of the analytes) and satlins transfer function in the output layer.

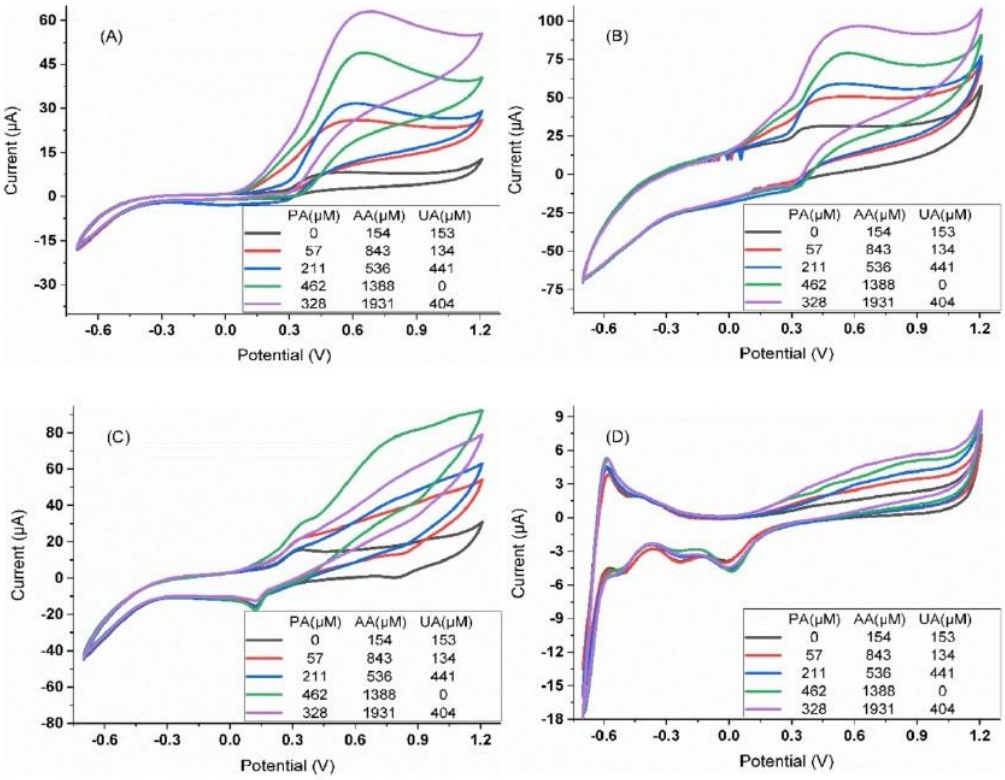

Figure 5. Representative voltammograms obtained for certain arbitrary mixtures of the different APIs (the concentration for each compound is indicated in the legend) with the four-sensor selected array: GECs modified with (**A**) ZnO, (**B**) PPy and (**C**) Prussian Blue, and (**D**) the metallic Pt electrode.

The comparison graphs of predicted vs. expected concentrations as well as the fitted linear regressions for the three determined species for the chosen ANN model are shown in Figure 6. As can be seen, a very satisfactory trend is obtained for all the cases, with regression lines almost overlapping or very close to the ideal ones. In order to better evaluate the goodness of the comparison, the regression parameters were also calculated and are summarized in Table 2. The obtained values are very close to the ideal values of slope (1), intercept (0), and correlation coefficient (1).

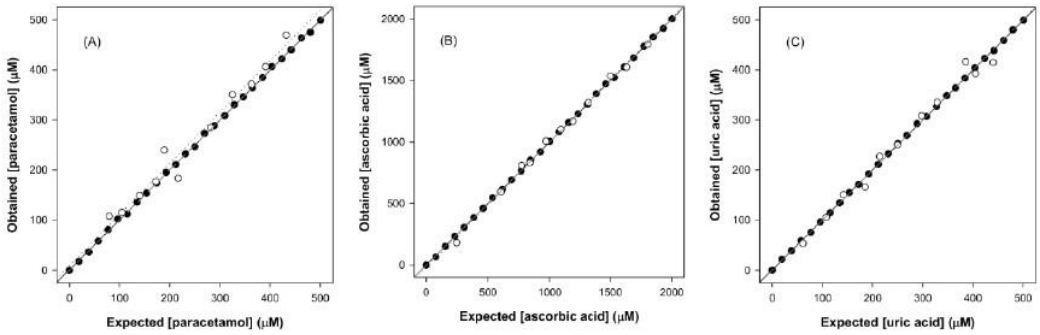

Figure 6. Modeling ability of the optimized DWT-ANN. Comparison graphs of obtained vs. expected concentrations for (**A**) paracetamol, (**B**) ascorbic acid, and (**C**) uric acid, for both the training (•, solid line) and testing subsets (○, dotted line). The dashed line corresponds to the ideal comparison line (y = x).

In this direction, to ensure that the former are statistically within the confidence intervals of the calculated regression parameters, the joint confidence intervals were calculated and plotted (Figure 7), as this allows to rapidly detect whether there are or not differences between the actual and predicted values at a certain significance level, judging simultaneously the goodness of slope and intercept [33]. In this case, the ideal point (1,0) is within the ellipsoidal confidence intervals for the three species, both for the training and testing subset, which allows us to state that there are no significant differences between the actual concentration and the values predicted by the model.

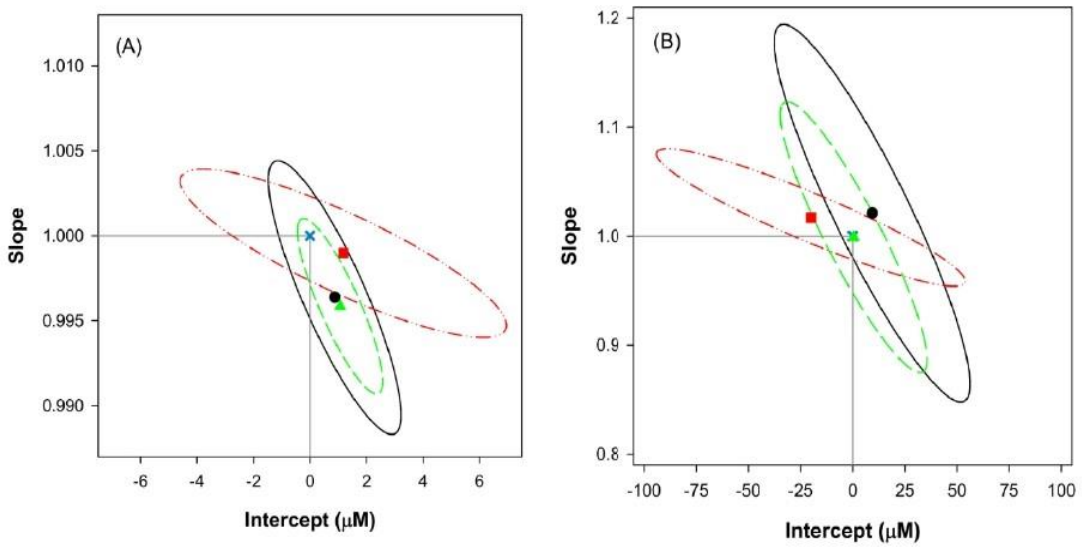

Figure 7. Joint confidence intervals for the three species: (•, solid line) paracetamol, (■, dash-dotted line) ascorbic acid, and (▲, dashed line) uric acid, and both for (**A**) training and (**B**) testing subsets. Also, the ideal point (1,0) is plotted (x); intervals are calculated at the 95% confidence level.

Despite the results for the training subset are more precise than those for the testing one, remarkable accuracy is obtained in both cases. The higher confidence intervals for the testing subset correspond to the usual behavior, which can be explained by two factors. On the one side, those samples are not used at all during the modelling stage, and consequently represent a more realistic metric of the model performance. On the other side, the lower number of samples of the testing subset in comparison to the training one results in higher tabulated *t* and F values that ultimately lead to higher confidence intervals. Similarly, the larger concentration range for ascorbic acid also results in a larger uncertainty for the intercept.

Finally, in order to benchmark the performance of the current proposed ET which is based on an optimized sensor array, its performance is compared to the one reported in previous works in which the same mixtures were analyzed (Table 2) [12,23,34]. To this aim, the root mean square error (RMSE) and its normalization (NRMSE) were also calculated to obtain a global metric of the system performance. Although some differences in the performance might be due to the different data treatment employed (e.g., PLS vs ANNs), it can be ruled out that a significant improvement has been achieved with the reduced sensor array. It can be seen that the smallest total NRMSE is obtained in the work reported here, but also, as could be expected from this, the improvement in the slopes and correlation coefficients is also highly significant.

Table 2. Reported values and current results of the fitted regression lines for the comparison between obtained vs. expected values for the different sets of samples and the three considered APIs. Intervals are calculated at the 95% confidence level.

Compound	Slope	Intercept (μM)	R^2	RMSE[1] (μM)	Total NRMSE[1]	Sensor Array	Ref.
training subset (n = 33)							
Paracetamol	0.942 ± 0.031	32 ± 21	0.968	[2]			
Ascorbic acid	0.933 ± 0.040	36 ± 25	0.947	[2]	[2]	Bare GEC plus metallic Pt and Au electrodes	[23]
Uric acid	0.873 ± 0.046	58 ± 25	0.923	[2]			
testing subset (n = 15)							
Paracetamol	0.895 ± 0.105	82 ± 71	0.848	[2]			
Ascorbic acid	0.919 ± 0.081	65 ± 41	0.908	[2]	[2]		
Uric acid	0.871 ± 0.138	−8 ± 86	0.753	[2]			
training subset (n = 33)							
Paracetamol	0.981 ± 0.032	13 ± 24	0.992	29			
Ascorbic acid	0.990 ± 0.031	6 ± 17	0.993	25	0.0257	Bare GEC plus metallic Pt and Au electrodes	[34]
Uric acid	0.981 ± 0.027	9 ± 16	0.994	23			
testing subset (n = 15)							
Paracetamol	0.990 ± 0.143	−2 ± 80	0.945	97			
Ascorbic acid	1.009 ± 0.136	−28 ± 78	0.952	66	0.101		
Uric acid	0.992 ± 0.208	36 ± 125	0.891	73			
training subset (n = 27)							
Paracetamol	1.000 ± 0.082	0 ± 25	0.962	29			
Ascorbic acid	1.000 ± 0.089	0 ± 25	0.955	31	1.00	SPCEs modified with CoPc, PB, graphite and CuO	[12]
Uric acid	1.000 ± 0.104	0 ± 31	0.940	36			
testing subset (n = 12)							
Paracetamol	1.021 ± 0.219	−13 ± 28	0.915	32			
Ascorbic acid	1.073 ± 0.422	−3 ± 54	0.762	71	1.03		
Uric acid	1.044 ± 0.334	−32 ± 36	0.829	44			
training subset (n = 27)							
Paracetamol	0.996 ± 0.006	0.9 ± 1.9	0.9998	2.43			
Ascorbic acid	0.999 ± 0.004	1.1 ± 4.6	0.9999	5.86	0.00378	GECs modified with ZnO, PB, and PPy plus Pt metallic electrode	This work
Uric acid	0.996 ± 0.004	1.1 ± 1.2	0.9999	1.64			
testing subset (n = 11)							
Paracetamol	1.021 ± 0.134	9 ± 36	0.971	26.4			
Ascorbic acid	1.017 ± 0.049	−20 ± 57	0.996	31.2	0.0368		
Uric acid	0.999 ± 0.096	1 ± 27	0.984	16.2			

[1] RMSE: root mean square error; NRMSE: normalized root mean square error; [2] data not available; GEC: graphite epoxy composite; SPCE: screen printed carbon electrode.

4. Conclusions

The application of a simple methodology for the selection of the optimal voltammetric sensor array prior to carrying out a quantitative application has been demonstrated. The proposed approach is based on the combination of PCA/CVA to assess the cross-response of the different sensors with the F factor to numerically carry out the selection of the sensors from the scores plot.

To illustrate the potential of the methodology, the discrimination and quantification of three different APIs have been demonstrated. In this work, we initially selected an array of eight different electrodes, and from those, only four sensors were selected for the quantitative application. Next, the performance to carry out the quantitative determination of the three APIs was attempted by building a DWT-ANN model. The performance of the model was very satisfactory, and huge improvement was observed when benchmarked against other reported ETs attempting the quantification of the same mixtures. This confirms the potential advantages derived from the current approach, which allows the a priori selection of the potential best sensor array based on its cross-response features, the implicit

reduction of the number of sensors (with the added advantage of instrumentation simplicity), and an improvement in the modelling performance (without requiring a posteriori pruning of the most relevant sensors).

Nevertheless, despite the good performance shown here, it has to be considered that there are many other clustering indexes, and that those are not universal. Therefore, future work has to focus on the comparison between different indexes and the suitability for different applications.

Author Contributions: Conceptualization, M.d.V.; methodology, M.d.V. and X.C.; software, X.C.; investigation, M.S. and N.R.; writing—original draft preparation, X.C. and M.S.; writing—review and editing, M.d.V.; funding acquisition, M.d.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Ministry of Science and Innovation, MCINN (Madrid) through project PID2019-107102RB-C21C. Manel del Valle thanks the support from Generalitat de Catalunya through the program ICREA Academia. Munmi Sarma thanks the support of the Government of Catalonia Secretariat for Universities and Research of the Ministry of Economy and Knowledge.

Conflicts of Interest: The authors declare no conflict of interest.

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