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Pharmaceutical Chemistry
Master's Thesis

DEVELOPMENT OF AN ELECTROCHEMICAL SENSOR FOR THE DETERMINATION OF MELAMINE BASED ON MOLECULARLY IMPRINTED POLYPYRROLE MOLECULAR RECEPTOR

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Development of an Electrochemical Sensor for the Determination of Melamine Based on Molecularly Imprinted Polypyrrole Molecular Receptor

Abstract

By

SAMSOM ARAYA FISSEHA

Food protection is doing for public health, economic growth, and tourism. In 2008, milk powder adulteration with melamine caused severe kidney problems for infants and children. Conventional methods for chemical detection are known, but they are not selective and time-consuming. Consequently, the development of specific analytical tools for melamine is necessary.

Molecularly imprinted polymers have been coming out in the past decade due to their long-term mechanical stability and selectivity. A novel electrochemical sensor and a simple approach for determining melamine have been devised, with polypyrrole used to capture the target analyte. The glassy carbon electrode has been functionalized with pyrrole and gold nanoparticles to enhance the signal. MIPs are cheap, easy to synthesize, and do not require an expert to operate. The MIPs formation complex with melamine has been assessed, and non-covalent weak interaction bonds, including hydrogen bonding, hydrophobic, and Pi-stacking, were included. Melamine in the MIPs sensor inhibits the oxidation of polypyrrole, resulting in a lower current than NIPs (Non-Imprinted Polymer). The reduced current is equivalent to melamine content observed within the sample and extends into the (0.1 to 5 μM). We can then use it for the detection of melamine.

The sensor was designed specifically for the usage in an aquatic environment with good reproducibility and stability with a covariance coefficient of 3.75% but is also highly selective compared to melamine–like analysts like caffeine, Xanthine, and Theobromine. This developed electrochemical sensor based on MIPs for melamine determination showed excellent sensitivity within 0.1-1.0 μM. Least square line and I (MA) = 35.3 logC+56.53(-log C)(μM), Where C, Melamine concentration and r = 0.9957 is the correlation coefficient. The computed LOD and LOQ for the MIP Sensor are 90 and 280 nM, respectively.

Contents

Introduction

"Melamine (MEL) is a synthesized polar organic compound with a cyanamide 1,3,5-triazine skeleton used to make various plastic goods"[1-2]. "It is a non-protein chemical molecule that contains around 67 percent nitrogen by mass, which is why it is known to be added to milk and other food items to artificially increase the protein content entice the consumer"[3]. The public health and powdered sector have suffered from milk adulteration. As a result, it is critical to keep an eye on the Melamine contamination in baby formula and other milk-based products using appropriate and sensitive analytical instruments for melamine identification.

"Chromatography became popular in the pharmaceutical, food, and chemical sectors, but the food industry utilizes it to offer qualitative and quantitative information on food compositions. Chromatography methods are the most routine analytical methods for detecting chemical contaminants or hazards in food still known to be conducted by Liquid chromatography (LC) and Gas chromatography (GS). "Since both chromatography feature powerful detection instruments that interact with columns, such as fluorescence detector FLD, Mass spectrometry (MS), and photodiode array detector (DAD), sample preparation and purification pretreatment are essential before chromatography analysis. However, conventional identification methods, like DAD and FLD, may neither be sensitive enough to meet the requirement of MRLs (Maximum Residue Limits) nor differentiate structural analogs. As advanced analytical technologies, LC-MS, GC, or GC-MS (gas chromatography-mass spectrometry) have been widely investigated to determine chemical contaminants in foods"[4]. Nevertheless, the detection of chemical hazards using chromatography is not suitable and is very time-consuming; advanced equipment requires expert personnel to run or operate.

New methods based on the antibody and enzymes also have been developed to determine food, chemical contaminants, and other critical metabolite residues. For instance, enzymes and immunoassay biosensors are simple and comfortable; neither instrument is needed, but these biologically based methods have drawbacks. They only immobilize protein biological interests, including enzymes and macromolecules; they lack binding sites for protein compounds, such as pesticides and food pollutants. Developing affordable, selective, high-sensitivity detections form on MIP achievable, while working electrode for MIPs/NIPS has been functionalized using Poly pyrrole.

CHAPTER 1: Food security requirements quality management food items

Food protection is paramount for public health, economic growth, tourism, and trade. Unsafe food presents a serious risk to children, pregnant women, and newborns. As the world's population is still growing and the demand for food like milk powder for children and infants, therefore monitoring and improvement should be necessary. As a result, they are linked to improving people's well-being. The food sector is rapidly upgrading its quality control, and nutritional safety is a significant concern. In more robust and broader processes to maintain food safety, assurance and administration conformity with international statutory requirements are required. "Current food enterprises and industries (such as minimally processed foods, altered meals, exceptional attention to dietary formulations, and other items with little or no additives) raise customer knowledge and desire for healthily processed or manufactured foods. For example, the 2008 melamine issue wreaked havoc on the Chinese milk sector and instilled panic among consumers[5]. In terms of food safety, it is essential to evaluate substances in challenging environments. Such as the nutritional value of processed foods, artificial ingredients, Microbial toxins, food additives (e.g., mycotoxins and endotoxins), antimicrobial drugs, pesticides, chemicals, and insecticides in farm products (vegetables, cereals, And so on).

"The food and Agriculture Organization (FAO) and WHO urge all countries to strengthen their food safety systems and be vigilant with food producers and traders" [5]. "Food quality and safety have been gaining prominence, mainly because of increased public awareness and expectations" [6]. "Consequently, local and international authorities have continued to raise the number of regulations and directives that must be followed to ensure that traded food products fulfill safety requirements before reaching the final consumer" [6-7]. "What consumers identify with food quality and safety in general, i.e., what they mean when they say a product is of decent quality or is safe to ingest, requires further investigation" [8]. Because they are regarded as "credence traits" the words "quality" and "safety" are difficult to define (i.e., product attributes the consumer cannot verify). Different things may impact customer value and protection, which might be built-in (such as the product's appearance) or extrinsic (such as the product's packaging) (e.g., a quality label).

"According to the World Health Organization's most current report "[5], around "600 million people worldwide become unwell after eating contaminated foods each year, with 420,000 deaths, resulting in approximately US $110 billion in productivity and medical bills" [9]. "Even in the United States, which has one of the best food safety systems in the world, the Centers for Disease Control and Prevention (CDC) estimates that 48 million people (roughly twice the population of Texas) get sick from foodborne diseases each year, with 128,000 hospitalizations and 3,000 deaths "[10].

There are ways to cause "foodborne sicknesses and food contaminants to enter the food supply pipelines during the preparation, processing, storage, packaging, and adulteration to increase the product value and content. Based on the nature of food hazards, they are classified into four primary categories, namely biological, chemical, physical, and allergenic hazards. Natural hazards cause most recorded foodborne outbreaks (bacteria, viruses, and parasites)"[11]. "On the other hand, chemical risks frequently create more severe health concerns than biological hazards, disturbing body metabolism, causing cancer, destroying DNA, modifying organ functions, and impacting reproduction and development" [12].

For instance, "melamine can cause or increase the possibility of "gallstones" in infants and children. According to the origins of chemical contaminants present in foods, food, chemical hazards are commonly divided into five categories "[13]:

• Pesticides (insecticides, herbicides, and fungicides) and veterinary medications (e.g., antibiotics, hormones) are used to kill, repel, or control specific types of plants and pests, while veterinary pharmaceuticals (e.g., antibiotics, hormones) are used to control or prevent diseases in animals.
• Natural toxins: include mycotoxins produced by fungi (e.g., aflatoxins, deoxynivalenol, ochratoxin A, fumonisins, Etc.), marine toxins (e.g., domoic acid, brevotoxin, etc.) accumulated in fish and shellfish, and some other toxins existed in food commodities.
• Natural and environmental contaminants: chemicals that accidentally or deliberately enter the environment. Ecological pollutants include arsenic, mercury, cadmium, lead, bromates, dioxins, furans, and polychlorinated bisphenols (PCBs).
• Deliberately added contaminants: chemical substances added into food products during manufacture to cause harm to consumers, such as melamine.
• Process and storage-derived contaminants: undesirable chemicals formed during processing or storage because of reactions between compounds that are natural components of the food. Examples of chemical contaminants formed during food processing include acrylamide, ethyl carbamate, furan, etc.

As a result, designing and developing simple and sensitive analytical methods to identify melamine for food safety is critical. "Spectrophotometry, colorimetry, Luminescence quenching, fluorescence, chemiluminescence analysis, surface-enhanced Raman scattering, gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), comprehensive micellar electrokinetic chromatography (SMEC), and especially naked eye sensing with gold nanoparticles as color indicating reporter are currently used" [14].

Significant advances in analytical technology have increased the speed of analysis and the ability to quantify contaminants. Significant advancements in monitoring foodstuffs and detecting harmful pollutants require fast and accurate analytical tools [15]. "These technologies can detect melamine levels below the legal limit in food. Still, they are costly and difficult to use, lack lengthy analysis, cumbersome sample readiness and portability, and are too necessary for professional personnel to do the calculation. There is a substantial gap between community needs and available methods. "Electrochemical sensors have been proven to be the ideal alternative for filling in the gaps and solving the difficulties of traditional approaches" [16]. As shown in figure 1 below, this gives firms and technological domains credit for safety and quality.

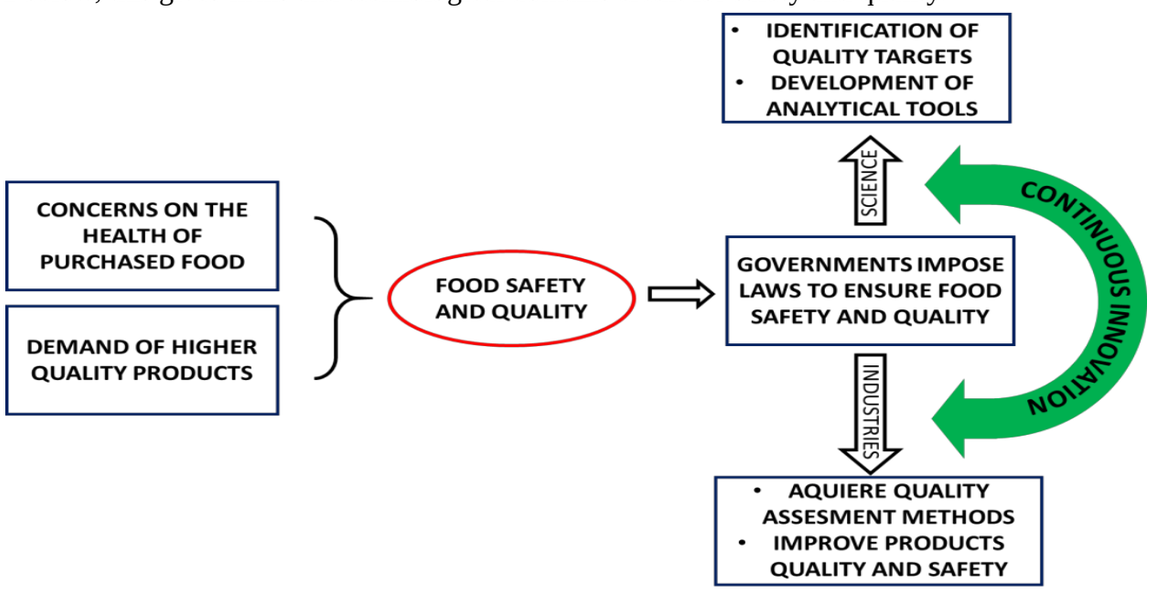


Figure 1: Usefulness of company quality, safety, and diversity of food

Escalating demand and requirements of the government regions such as the European Union, the FDAsof USA, and China's cooperatively enhanced customer understating encourage better control of processed food products. "It is not a simple task since the foodstuff contains many inactive additives. Therefore, the demand for quality control for food products increases

because of the established food hygiene assurance system based on cost-effective procedures, regardless of matrix complexities. The possibility of preconcentration and detection of analytes typically presents at trace levels"[17]. Compounds are essential to ecology; however, they can cause bioaccumulation. However, they are unreliable due to numerous food constituents' influence and the dispersion of meal supernatants. Therefore, it was evident that it needs material with high specificity and selectivity for sample treatment is increased interest, and molecular imprinted come in response to the above demands.

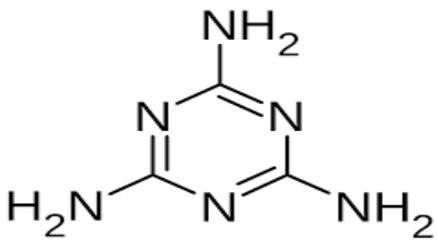


Figure 2: Melamine structure formula created by Chem Draw

"Multiple outbreaks of melamine toxicity have been shown in animals in 2004 and 2007, supporting the kidney failure hypothesis, and the exact toxicity of melamine mechanisms remains vague. Since that period, melamine has been considered safe for animals and humans worldwide. Pharmacokinetics studies in animals' melamine are deadly to 50% of rats at a dose of around 3200mg (about the weight of a penny) /kg" [18], and in rodents, its" half-life varies from 2.7 to 4.9 hrs. " [19] And "takes around four hours in piglets "[20].

(A) **(B)**

Figure 3: (A) adulteration in Milk [84] and (B) China 51,000 babies ill due to melamine milk in 2008[85].

For example, "melamine-contaminated animal feed caused renal failure and urological problems in dogs in Asia and North America in early 2004 and 2007 and received little attention. Still, over time, adulterations or illegally added melamine to milk powder to boost nutritional content yielded in China's melamine-contaminated milk powder crisis"[21]. "His actions affected the health of babies and children. Furthermore, getting attention in the world in 2008. According to the Chinese Ministry of health statistics, 294,000 were affected. (About half the population of Wyoming) Fifty-one thousand nine hundred children were hospitalized after being confirmed with At least six kids died because of melamine–related renal crystals "[22].

"Melamine formaldehyde resins produce laminates, coatings, and molded compounds for kitchen and dishware"[23]. "When coupled with resins, melamine is utilized as a flame retardant when associated with wax because it releases retardant nitrogen gas upon combustion or scorching" [24]. "Melamine was employed as early as the 1950s, and non-protein sources were used in ruminants because of their high nitrogen concentration. Many melamine-fed ruminants' kidney failure was recorded in 1960, demonstrating that melamine is a non-absorbable nitrogen source in these animals" [25].

1.1 Ultra-modern methods for the detection of chemical contaminants in food

1.1.1 Conventional methods for the detection of chemical contaminants

Nowadays, chemical hazards in foodstuffs are trace organic molecules present at low concentrations. Measuring the sensitive and accurate analysis qualitatively for the compound of our interest in foods can be incredibly challenging. The food contains a variety of chemical compositions of materials and is complex; once it is an adulterer with another chemical during the processing stage, the analyst pretreatment, purification, separation, and detection make it difficult. Best case scenario sample pretreatments are one of the most important that direct to satisfactory results interns of accuracy and precision physiochemical analysis. Conventional analytical methods give an example. "Thus, liquid-solid extraction and liquid-liquid extraction are commonly applied to remove the debris and interference (e.g., Fats and lipids), take care of the sample by increasing the concentration and convert the workable sample form determination. Nevertheless, the complex nature of the chemical methods for preparing food analytes still lacks specificity toward different detection targets and is time-consuming. It may not be able to assure quantification accuracy" [26].

A few refined or enhanced approaches for identifying pollutants utilizing examination in a research laboratory are presented. Such as the "QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method [27], Solid-phase microextraction (SPME)"[26], etc., have been introduced, and they have been designed to improve the overall separation efficiency. However, they fail to recognize and separate the analytes.

1.1.2 Brief overview of molecularly imprinted polymers (MIPs)

Molecularly imprinted polymers (MIPs) along with NIPs (designed without imprinted target analyte) are synthetic analogues of natural, biological antibody-antigen; they employ "lock and key" idea attach target compound during production selectively. " We make the lock based on the sample and then look for the secret to rebind the template. The conception of "MIPs is creating a well-defined three-dimensional pocket or cavities innermost of the polymer matrix, comparable in solid size, form, and structure, particularly the functional groups of the compound of interest. MIPS imaginably provides biological receptor precision and selective with clear precedence of longevity concerning environmental conditions and is inexpensive compared to the traditional analytical methods. Only used recognition elements include enzymes, antibodies, and aptamers" [28].

"According to Chemical Abstracts, the first publication describing an "imprinted polymer" was published in 1984 in Lund by K. Mosbach and B. Sellgren" [29–30]. "The phrase "imprinted polymer" was initially used in a paper by G. Wulff in 1985" [31], but "Wulff had been publishing articles as part of a package called "Enzyme-Analog Built Polymers" timely 1973" [32]. The preceding study produced separate parts of science. The Mosbach lab concentrated on working with non-covalent chemical bonding, whereas Wulff favored covalent bond imprint. The most noticeable contrast joining both pair techniques is the pure science forced to separate templates of polymer.

Significantly, "covalent synthesis" should result in a more uniform collection of cavities rebinding and, eventually, more key target MIPs. These groups' research started in the late 1990s and has resulted in studies on various uses of this technology. Mosbach"[33–36] and "Sellgren described emerging separation and sensing materials; Wulff "[36–40] used to imprint polymers extensively in catalytic reactions, as evidenced by the heading of his beginning chain articles; and "K. Shea, beginning in 1993, reported on the unique biomedical application, such as a plastic antibody inserted into living mice. Although articles describing the manufacture of MIPs, by this

author, appear in the Russian literature as early as 1989, the phrase molecularly imprinted polymer sensor seems to have been first used by S. Piletsky in 1992 "[41].

Nevertheless, as we know, biological compounds suffer from "physiochemical stability, and they are not suitable for application in harsh environmental conditions (e.g., extreme temperature or pH)" [42]. While MIPs, based on a polymer that needs to be used for the processing of MIPs, can usually be stored for a more extended period, and therefore they do not require specific e. It can be held in various environments at a variety of temperatures. As a result of its selectivity to analyte molecules and the higher physical and chemical stability, they got an increasing interest in researching in different fields, including the area of detection and drug delivery systems" [42]. MIPs has obtained a research interest, and the trends still skyrocketed in publications published to see the directions below.

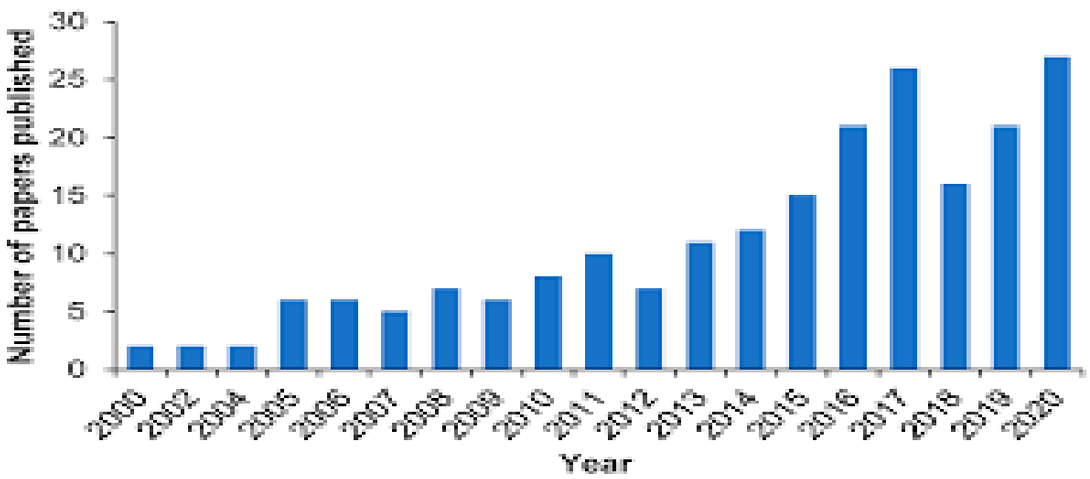


Figure 4: MIPS number of a paper published in the last 20 years [82].

1.1.3 MIP preparation methods

1. An analyte is utilized to improve molecular recognition as a MIP (Molecularly imprinted polymers) template. During the process of polymerization, there are two different monomers necessary. These are.
2. The conducting monomer is in charge of making a bond with a target.
3. Intern connections help form a 3D dimensional matrix and rigidity to the polymers.

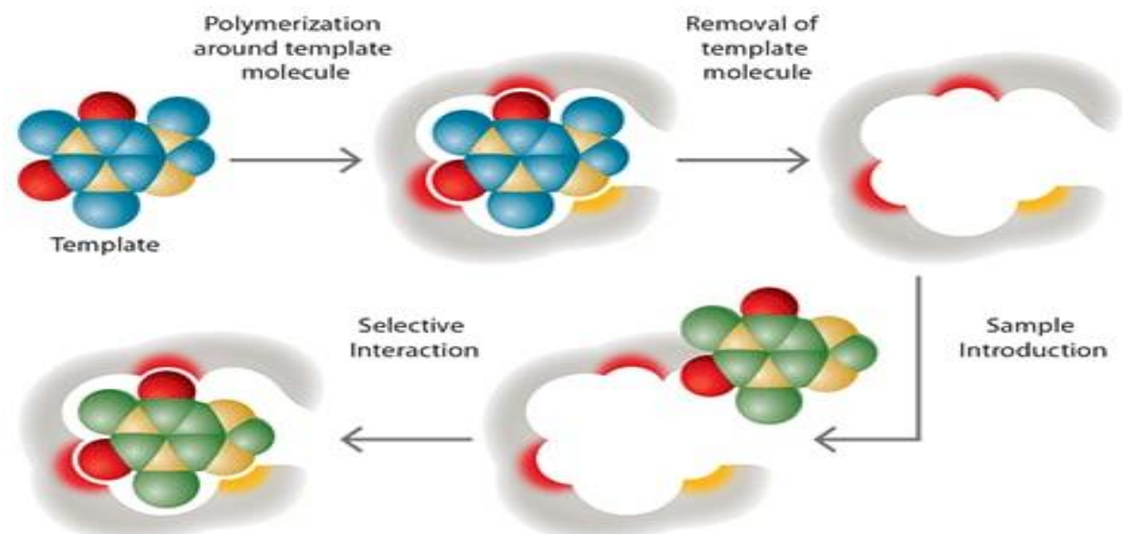


Figure 5: Depiction and theoretical illustration MIPs [81].

"There are three main electro polymerization synthetic procedures or steps that can be prominent. There are three main electro polymerization synthetic procedures or measures that can be prominent. Allow us to begin with the first step. Let us start with the first step.

1. Prepolymerisation stage: The target (analyte) will react with them to generate a combination before polymerization with a template molecule. Here, during pre-polymerization, there are two ways of interaction which are covalent and non-covalent interactions. Covalent interaction is an e effective molecule, and the analyte sets up a robust chemical connection., while non-covalent interaction is weak bonds like hydrogen, ionic and hydrophobic interactions. These types of interactions have more interest in the field of detection with comprehensive imprinting advantage and fewer restrictions on functional groups "[43].

2. Co-polymerization stage: "The functional monomer is copolymerized with a crosslinker in the second step, which is activated by heat or photochemical activation of an initiator" [44-45].

3. Extraction or template removal step: after the formation of the complex matrix in this process, the template will be removed with a suitable solvent, and the polymer will leave cavities similar complementary groupings by size, form, and activity (memory of the analyst) it is the MIP sensor.

"Compared to the biological analogues, enzymes, antibodies, peptides, and proteins, MIPs have superior advantages in the stability, reusability, inexpensive, easier regeneration, sterilizations, and a longer lifespan. A variety of synthetic procedures such as bulk, high dilution, emulsion, and solid-state polymerization are only a few of the most popular techniques that have been presented"[43]. In this thesis, I used the electro polymerization technique to give simplicity and uniformity with electroactive surfaces for better charge transfer and control of the film thickness formed on the working glassy carbon electrode surface.

Optimization synthetic procedures for the formation of high selective It is possible to obtain imprinted material. The two most important parameters that affect the MIP's quality are before complex construction and the proportion of crosslinker to functional monomer. Most problems encountered during the MIP's preparation come from failure to create a complex between the active subunit and the target molecule, leading to weak affinity, non-homogeneity, and less selectivity for recognition.

"Selecting a stable, functional monomer to create a compound with the template is vital. Therefore, different computational approaches and modeling are available" [46-47]. For example, "the ratio and the rigidity in the thermodynamics of the prepolymerisation complex can be assessed by isothermal titration calorimetry"[46]. "A choice of the other titration methods can be executed by UV-Vis, Fluorescence, or NMR spectroscopy" [47].

The strength of the synthesized model has affected aside "proportions of the functional monomer and crosslinker. The formulation for the polymer matrix has a substantial impact on the MIPs' capacity to recognize them. As the crosslinker increases the rigidity of the polymer matrix "[48], the crosslinker affects the extraction and incubation of the template. Consequently, because alternative molecules cannot adjust to this location, the detecting hole will be more exclusive to the template.

The final MIP template will be obtained by carefully selecting the monomer, proper formulation, and optimization of the synthesis parameters using trial and error. "The most critical parameter MIPs sensors are the imprinting factor (IF). These parameters are essential to characterize the imprinting process comparatively if the motif is not present during synthesis. Binding capacity (BC) is calculated as the ratio of the strength(concentration) of the target molecule deposited from the solution divided by the initial concentration of the solution

multiplied by the balance of the template binding in the MIP to the NIP (Non imprinted polymer)"[49].

1.2 MIPs for various applications

"MIPs is highly selective binding affinity to their template (analyst). Moreover, have a wide application in different fields, such as drug delivery"[50], "compounds with important biological effects vitamins"[51-52], "chromatographic separation"[53], "purification of biological and chemical reagents"[54], carcinogens compounds [55], in environment and foods analysis [56], flavoring compounds [57-58]. MIPS in food analysis can be used to separate materials to selectively distinguish and concentrate trace amounts of a target substance from complex analogues of food matrices. For instance, "molecularly imprinted solid-phase extraction can be employed as an adsorbent for solid-phase extraction"[59-60]. "MIPs have been applied as stationary phases in chromatographic techniques, such as HPLC (High-Performance Liquid Chromatography), thin-layer chromatography (TLC), and capillary electrochromatography (CEC) "[61-62].

MIPs can also be used as a specific recognition, sensing target, and other sensing elements for chemo sensors. "MIPs-based sensors can selectively bind with the target analyte and induce transduction to the transducer to generate an output signal for detection; such sensors are called electrochemical sensors"[63].

"MIPs can gather qualitative and quantitative information for the target analyte different classes, including macromolecules, drugs from pharmaceutical dosage forms of life sciences biological serum samples can be traced. Both optical sensors based on surface-enhanced Raman spectroscopy (SERS) and quantum dots-based fluorescence sensors have some distinct precedence for selectivity, sensitivity, and Chemical pollutants in food can be detected quickly" [65]. MIPs have numerous medical applications, including drug delivery systems, oncogene biomarker testing, environmental monitoring, chemical detection, etc.

1.3 Chemical sensor technology

"According to the international union of Pure and Applied Chemistry, a chemical sensor is defined as "a device that transforms chemical information, ranging from the concentration of a specific sample component to the total composition analysis, into an analytically useful signal" [66].

"Generic sensors can be classified into three essential components:

- The signaling component (responsible for the analytical signal when the analyte is recognized)
- The recognition unit that binds to the analyte
- The transducer that connects the two units

For instance, a type of signal generation is described here "[42].

- **The Electrochemical:** The current or capacity is measured when it changes.
- **The Optical**: Change in the light scattering, fluorescence, refractive index, and absorbance measurement will be taken.
- **Mass sensitive**: Piezoelectric, or acoustics wave devices, a slight change in abundance when target binding alters the vibration regularity about top sound.
- **Magnetic property:** The presence of the analyte causes a changing magnetic field characteristic.
- **The Heat:** swap in hotness can be counted while indicating indicator(sign).

"Varieties of signaling units are essential, but out of these, both the electrochemical and optical devices are the most studied, low cost, easy to miniaturize, can be portable, have good sensitivity

and low interference, and have a wide application in food chemical determinations' attach to particular targets and provide an optical response or an electrochemical signal. I want to discuss the sensors' construction, sensing principles, and signaling pathways mechanisms and their applications in food assessment, biosensing, medical diagnostics, and innovation"[66].

1.4 Electroanalytical sensor apparatus

"An electrochemical sensor is a device that converts an analyte's contact with a receptor on the electrode's surface into a proper analytical signal" [66]. "Electrochemical sensors are widely employed in various fields, including environmental, industry, transportation, and medical monitoring, to analyze metabolism and watch biological processes. These electrochemical sensors are inexpensive in cost, high sensitivity, and powerful tools to gain real-time information or brief analysis time for monitoring in situ observations for process control of the composition just without sampling. Traditional electrochemical sensors utilize aqueous or another liquid electrolyte and run at temperatures of up to 140°C, while solid electrolytic sensors work at up to 500°C. Electrochemical sensors can be employed in definite matrices at temperatures ranging from 30 to 1600 degrees Celsius, depending on the electrolyte "[67].

Amperometry, Impedimetric, and potentiometry are electrochemical principles and measurement assumptions. The temperature, atmospheric conditions, and chemical nature influence the measurement circumstances influence sensors' sensitivity, specificity, and stability (reliability and durability).

"Electrochemical sensors, for example, at low temperatures, are used to determine pH, conductivity (impedance), and hence the number of ionic species and gases. Solid electrolyte sensors monitor various components in exhaust gases and molten metals at elevated temperatures. Sensors are used in many technical processes to determine oxygen gas, equilibrium oxygen in reducing gases, and dissolved oxygen in metal melts"[67]., Commercially available, for example, in the steel, ceramic, cement, and glassmaking industries. Because of their strong affinity for the target molecule, molecular imprinting techniques for the electrochemical detection of diverse analytes have experienced significant advancement in recent years.

Potentiometric and amperometry sensors are the most common types of electrochemical sensors. State of the art in sensor technology and its applications are characterized by:

- New and improved electrochemical sensor materials like AuNP in MIPs sensors are being developed to improve sensitivity, selectivity (specificity), and stability.
- Evolution of sensor for high and low-temperature implementation or applications, e.g., pH measurement
- Develop sensor/biosensor build from MIPs to detect hazardous contaminants, e.g.,
- Development of sensors/ biosensors based on biological principles, e.g., Covid-19 sensors for sensing covid spike virus
- Development and extension of sensors that tolerate high pressure, temperature, and aggressive acidic and basic media. The current flow is measured in voltammetry as the potential is linearly swept or changed. When the potential is obtained, the analyte undergoes oxidation/reduction, and an indirect current is recorded.

For performing this experiment, "three electrodes are needed:

1. The analyte's redox reaction took place on a working electrode.
2. A Reference Electrode is a device that measures the potential difference between the working electrode and it.
3. A counter electrode measures the current flowing between the counter electrode and the working electrode.

The potentiostat oversees varying the potential and measuring the current; the data were recorded on the computer for process and analysis. See below the schematic diagram for the electrochemical setup"[67].

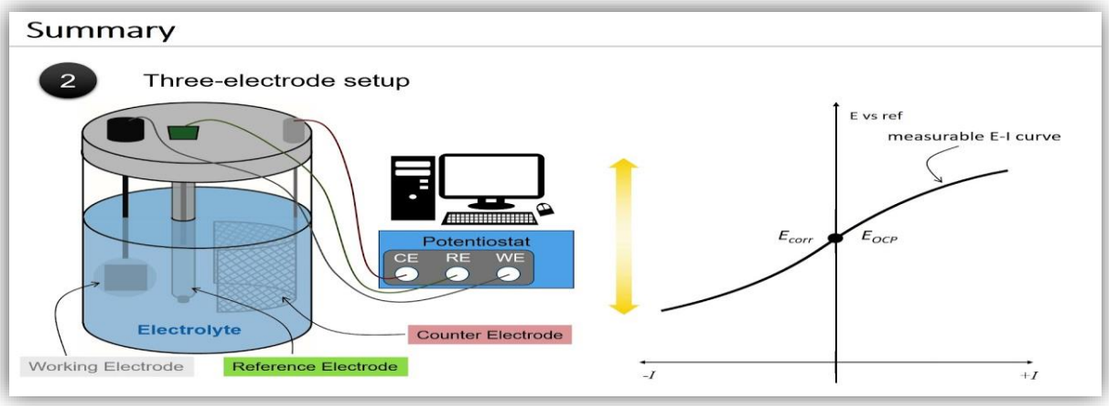


Figure 6: Standard 3- electrode setup connected to potentiostat and Galvano stat with a measurable potential-current curve.

"By redox reaction, the target molecule could reach the surface electrode. Voltammetry and DPV in supporting electrolyte solutions are proven in this thesis, and charge transfer is carried out through diffusion; it is 5.569*10-3mC. Consequently, the amount of compounds at the top of the conductor surface is initially compared mass solution's amount (figure 6). Figure 6 shows the oxidation process.

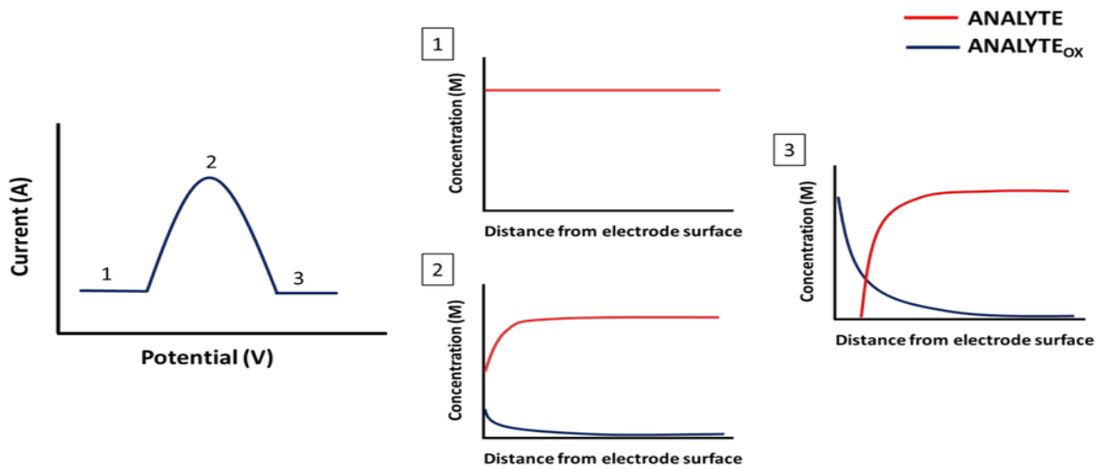


Figure 7: The Diagram shows analyte concentration and oxidation with distance from the glassy carbon electrode. Drawn by Origin Pro2022. Figure 7.1: Potential for inadequacy in beginning the response. The concentration in the sample mixture is equal at the top electrode. Fig. 7.2 When the true potential is attained, the content of the target near the electrode falls, and no current is produced. Figure 7.3.

Voltammetry measurements were performed in this thesis, and the experiment was conducted using an electrochemical technique. In this analysis, the electric flow is the concentration of charge transported through the redox process, consequently the amount of target in the mixture:

$$Analyte_{red} \rightleftharpoons Analyte_{ox} + n\ electron$$

Scheme 1.0 general redox reaction process

Voltammetry measurements were performed in this thesis, and a calibration curve based on the current intensity vs. target concentration was generated, which may be utilized for

analytical reasons. Furthermore, in the "experiment, the region beneath the peak of each peak is proportional to the objective content of oxidized or reduced substances, and the peak potential is a feature of the redox process that is impacted by electrochemical material. This assists in determining the content of the analyte chemical. The use of various upgraded electrode materials, such as glassy carbon, carbon fibers, carbon nanotubes, and conducting polymers, influences the advancement of this technology" [68-70].

"Poly pyrrole, polyaniline, polythiophene, and poly (3,4-ethylene dioxythiophene) PEDOT have all been used to modify electrodes for the detection of physiologically active substances "[69-70]. As a result, finding a suitable material to use to alter the surface working electrode is critical; the material must be eligible for the potential required for the electrochemical process to occur and experimental and instrumental parameters that will aid in the recording of scientifically sound signals, such as signal to noise ratio, must be discovered through trial and error.

1.5 Core aims and objective

The aim of this research was to design melamine imprinted polypyrrole-based MIP using an electrochemical polymerization method. The goals are:

A. Design of MIP-based electrochemical sensor device-specific for melamine determination in real-time
B. Characterization and optimization of the device
C. Fundamental sample analysis for melamine

2.0 Experimental part

2.1 Reagents and Materials

Pyrrole 99.6 % was obtained from Alfa-Aesar, 1,3,5-Triazine-2,4,6-triamine, Gold Nano Particles (AuNP), Potassium chloride (KCl), Deionized water (DI) used in our lab thought out the experiment, Potassium ferricyanide, Potassium ferrocyanide, Sodium chloride (NaCl), Sodium Phosphate Dibasic (Na_2HPO4), Potassium Phosphate Monobasic (KH_2PO4), Sulfuric acid (H_2SO4), Potassium Hydroxide (KOH), Hydrochloric acid (HCl), were obtained from Thermo Fisher Scientific Alfa Aesar, Massachusetts, United States). Buffer solutions, the substances described above, were utilized in this research. At least analytical and Reagent grades were applied without further purifications. All solutions for cyclic voltammetry were prepared in 0.1M KCl+PBS, differential voltammetry was prepared in 0.1 M PBS, and Oxidation reaction was measured. Milk powder was acquired from neighborhood food shops in Vilnius, Lithuania.

2.2 Instrumentation and Apparatus

Cyclic voltammetry was performed on a Potentiostat/ Galvanostat Nova Auto lab 3526 Km (Utrecht, The Netherlands); an electrochemical workstation was a three-electrode cell with a platinum counter electrode and an Ag/AgCl (3MKCl) reference electrode. The working electrode was a glassy carbon electrode with a diameter of 3.0mm (about 0.12 in). The cell temperature was at ambient temperature (20°C). To assess the electrochemical response of the MIP and NIP coated electrodes, a 0.1M KCl electrolyte and a 0.1M PBS (pH 7.4) solution were utilized throughout the investigation. CV measurements were done in a Potentiostat/ Galvanostat Nova Auto lab 3526 Km Utrecht, The Netherlands, with a Scan speed of 0.1V/s within the –1 to +1V range. Several cycles were performed throughout seven of the investigations, then optimized by changing one parameter once during the polymerization process. Aqueous solutions were utilized with deionized water (DIW) (18.2M.cm) purified with clarity filtration equipment

2.3 Deposition of Conducting polymer based layer

Materials research and innovation are increasing quickly as these materials have been synthesized for many applications in electrical devices, biomedical engineering, nanotechnology, sensors, and biosensors. The electron acceptor and electron donor occurred as a host when electrons migrated from higher occupied to lower empty molecular orbitals, resulting in charge transfer conductivity.

The organic molecule should have conjugated double bonds or electron delocalization to enable charge transfer on the polymer. Heterocyclic organic compounds, such as nitrogen, oxygen, and sulfur, have also been investigated for charge transfer as thin film formation for conductivity, with Pi orbitals that participate in the overlapping for the coming electrons to transfer within the system, allowing oxidation and reduction, for example. Pyrrole is a conducting polymer that has been widely studied in the development of sensors and biosensors for analyte immobilization via weak interactions that keep the memory of the contacted target the same size and shape. Poly pyrrole is easily polymerized from pyrrole.

This thesis is used to immobilize melamine on the surface of the Poly pyrrole polymer. Electrochemical deposition is straightforward and affordable; potentiostats and galvanostats monitor the working electrode's film.

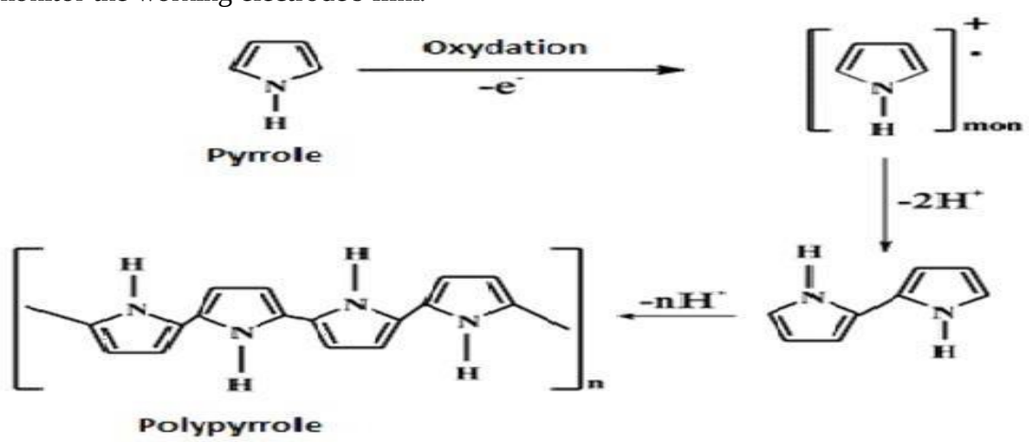


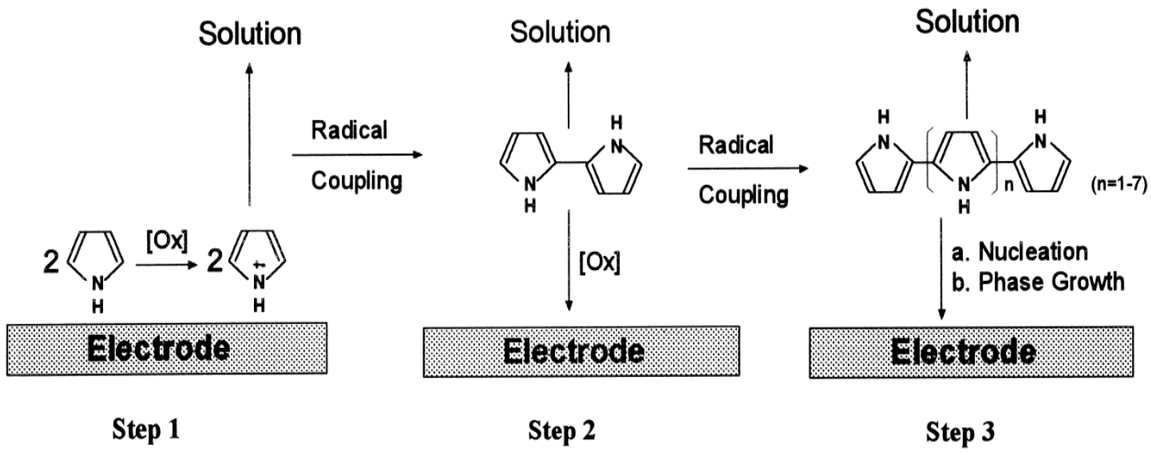
Figure 8: Electrochemical Polymerization of Pyrrole, by A.F.Diaz and K. Keij Kanazaw

2.4 Electrochemical polymerization of Pyrrole and formation of molecularly-imprinted thin films over electrode surface

Transform infrared (FTIR) spectrophotometry was planned to investigate the interaction of the monomer units Pyrrole and MEL to produce a pre-polymerization complex, but this was not done due to time constraints and a lack of equipment done. The working electrode (GCE) was cleaned using stock solutions (0.01 M potassium ferric cyanide + 0.1 M KCl solution) and polished clean with 0.3- and 0.05mm alumina slurry for 2 minutes. Then cleaned multiple times with water, following the reported normal processes, and touched up with tissue paper.

The electrochemical synthesis for the MEL-MIPs has carried out the following procedure: Melamine 1 mM was dissolvable with 50ml (about 1.69 oz) of PBS and mixed with 0.1M of pyrrole. A magnetic stirrer was used to agitate the liquid solution for 40 minutes at 20 degrees Celsius. The solution then was continually swirled for 10 minutes. Before electro polymerization, the solutions were maintained for around 1-2 hours to enable the complex between polymer matrix and motif to develop. MIP film was made using a monomer-to-template proportion of 10:1.

The MIP and NIP were directly settled on top face GCE by voltammetric cycling in 0.1M PBS solution in a typical three-electrode cell configuration.



Scheme 2: Electropolymerizing of pyrrole and depositing on Surface electrode [80], scheme reaction done by authors)

Seven consecutive voltammetric cycles were performed at 0.1V/s in the range of (-1.0) - (+1.0 V). After electrodeposition, the films were rinsed with water to eliminate any surplus monomer from the polymer film. Using potentiodynamic electro polymerization, a thin film is deposited on the surface of the working electrode. On the GCE, electrochemical methods were used to describe the film. The MIP was determined using the differential pulse voltammetry sensor's ability to detect and measure the MEL template, selectivity, reusability, sensitivity, and stability. All electrochemical tests were conducted in a PBS (0.1M Ph 7.4) and 0.1M KCl, with CV (Cyclic Voltammetry) measurement used for film characterization and deferential pulse voltammetry used for MEL detection and quantification. Electrochemical methods were used to characterize both electrodes NIP/MIP and demonstrate the polymer's production on the surface.

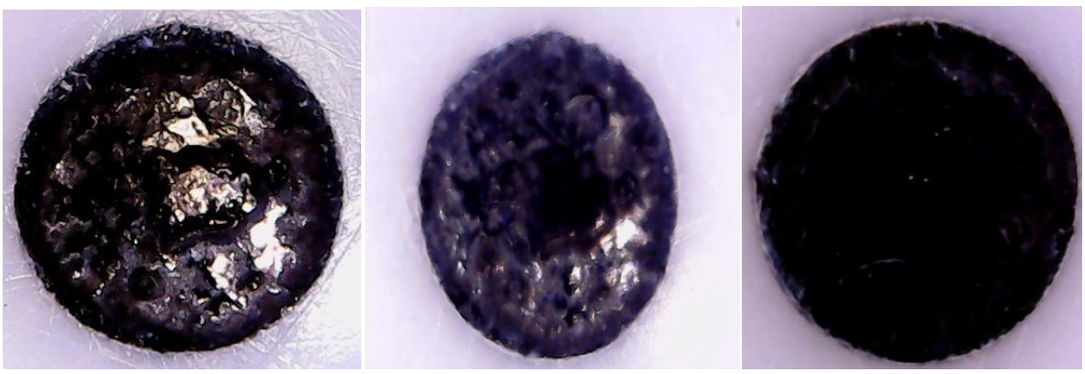


Figure 7: Bare GCE, NIP/MIP, During the electro polymerization and deposition experiment, a picture was captured)

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Our lab provided Reagent plus pipettes in various volumes, used to accurately add solution amounts less than 10 ml. The analyte binding experiment also used microliter Hamilton syringes (1mL) from Hamilton Company. Other than that, volumetric cylinders were used to measure liquid volume. Origin Pro 2022 software (Origin Lab Corporation) was used for data analysis, graphing, and fitting.

CHAPTER 3: Results and discussion

3.1 Poly Pyrrole characterization on a glassy carbon electrode

According to the electropolymerization procedure with optimum settings, the polymer film adheres easily to the exposed electrode's contact receptors. By watching how the current of several electrodes changed in 1 mM MEL, the effect of surface functionalization on the current approach of the glassy carbon electrode electroactive area was studied. The change in the recent redox peak to even more positive potentials shown in CV curves indicates that the template was engaged in polymerization (Figure 9). From bare GCE to NIP, the oxidation current maxima drop. Finally, MIP changes the surface conductivity from absolute conductivity to more undersized conductors. Poly pyrrole oxidation is more robust in the presence of MEL than in the absence of MEL, showing that the motif is exceptionally functional. Because the template is trapped in the polymer matrix and diffuses towards the electrode, MIP film has a larger current than NIP film. The modified electrode was electrochemically assessed after MIP/GCE fabrication with CV between -1.0 and +1.0 V at 0.1V/s in 5mM [Fe (CN) 6]-3/-4 containing 0.1M KCl until a consistent voltammogram was obtained. The electrode's current receptivity change was employed to corroborate the formation of the MIP surface coating. Set up the CV Plot.

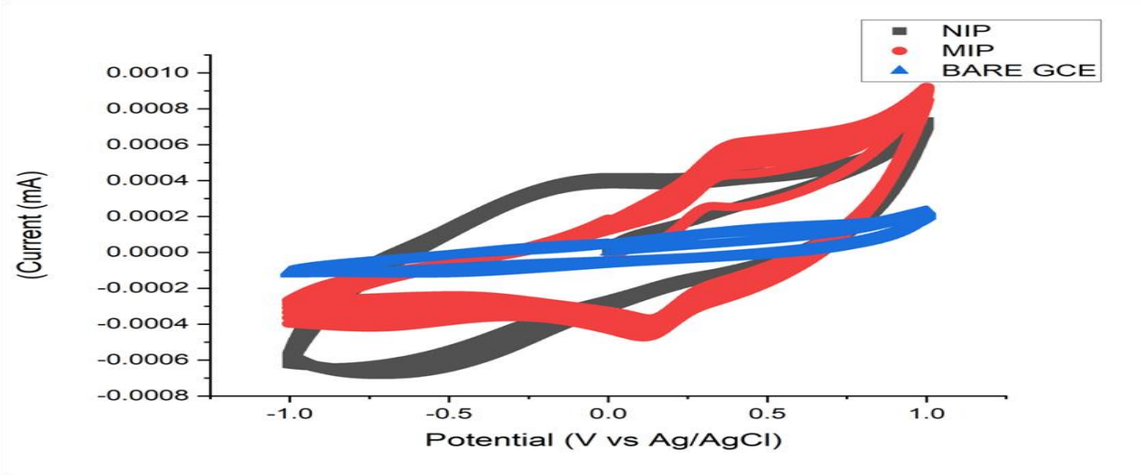

Figure 9: Response to cyclic voltammetry for characterization film formation on GCE, NIP/MIP, created by Origin data analysis.

3.2 Experiment interraction of melamine with the NIP and MIP

The binding characteristics of MIP and NIP were evaluated using a DPV. A holder was used to hold the glassy carbon electrode with MIP or NIP., which was linked to a 10ml (about 0.34 oz) electrochemical beaker incubated in melamine PBS solution for 10 minutes at various concentrations. The optimal contact time (i.e., 10 minutes) was determined by evaluating residual melamine in solution at different concentrations and times. A calibration curve was used to measure the concentration bound on MIP or NIP. Every sample was subjected to three separate measurements, with the mean concentration reported.

To make effective MIPs, it is critical to understand the interaction between the pyrrole and the melamine. Melamine contains three donor hydrogen bond units and six acceptor hydrogen bond units. While pyrrole only has one hydrogen bond donor. Many different intermolecular interactions between the Pyrrole and Melamine can theoretically occur. Hydrogen bonding, hydrophobic interactions, electrostatic interactions, and Pi - stacking are some of the methods used.

Quantum-mechanical approaches are beneficial for explaining molecule recognition using hydrogen bonds and other weaker contacts. Perturbation and computational chemistry are excellent tools for studying how organic molecules interact at interfaces; For such weak interactions, incorporating polarized and diffusion functions in the basis set must provide satisfactory results.

3.3 Optimization conditions and parameters

The kind of monomer, the target to polymeric matrix ratio, and during MIP polymerization, the pH all affect the performance of a MIP modified electrode. The stoichiometric ratio of the matrix towards the target has impacted the number of binding cavities accessible in the final MIP formed. Furthermore, multiple Pyrrole and MEL molar concentrations (10:1, 10:2, 10:3, 10:4, and10: 5) have been used to determine the best molar ratios. The selected proportion has been 10:1, which resulted in the elevated response of the current. The combination of materials is ideal for creating binding chambers (pockets) accessible and capable of holding the template. The MIP film's design extraction and linking were addressed for the optimum ratio. The oxidation current peak changed significantly after MEL extraction and capture. The flow of electrons through film improved by the diffusion of the film's template, which raised the recent rise.

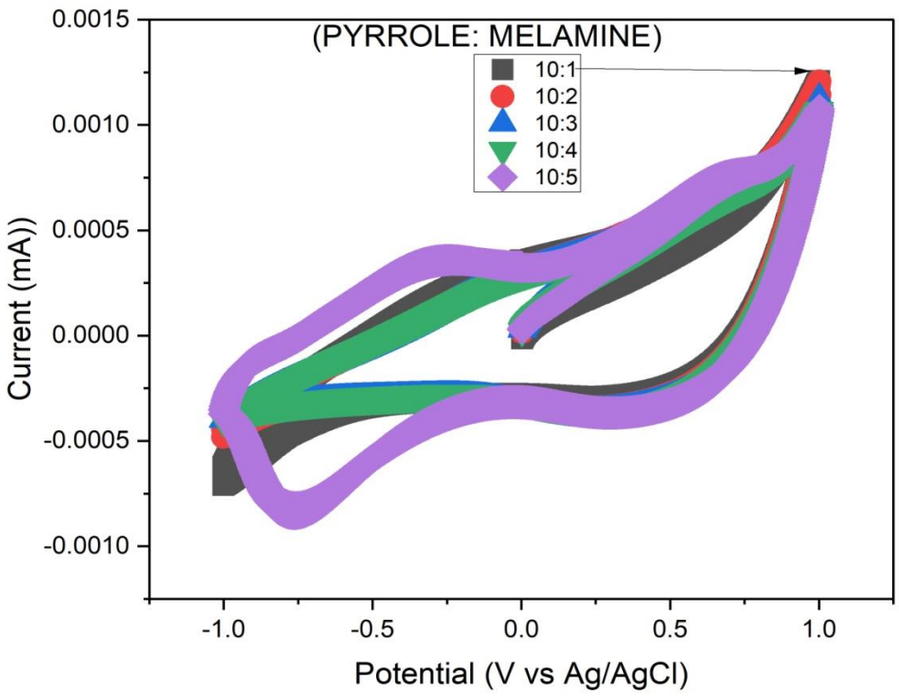

Figure 10: Optimization of Concentration, created by Origin data analysis.

The device's efficiency was evaluated using a variety of scanning speeds and cycles. Scanning rates of 0.05. 0.1. 0.2, 0.4, and 0.5 V/S were utilized with scanning cycles of 5, 10, 15, 20, 25, and 3. When 0.1 Micro molars, MEL rebounded in PBS at a 0.1 V/s swept rate. M considerable current flowed with a scanning cycle of seven (7).

Other parameters, in addition to electropolymerization conditions, must be carefully considered throughout the production of MIP sensors. The pH of the incubation buffer is one of these factors time required for template extraction and rebinding in the template PBS solution. The pH of the electrolyte solution influences MEL. MEL degrades in water with a pH of less than 7.4.

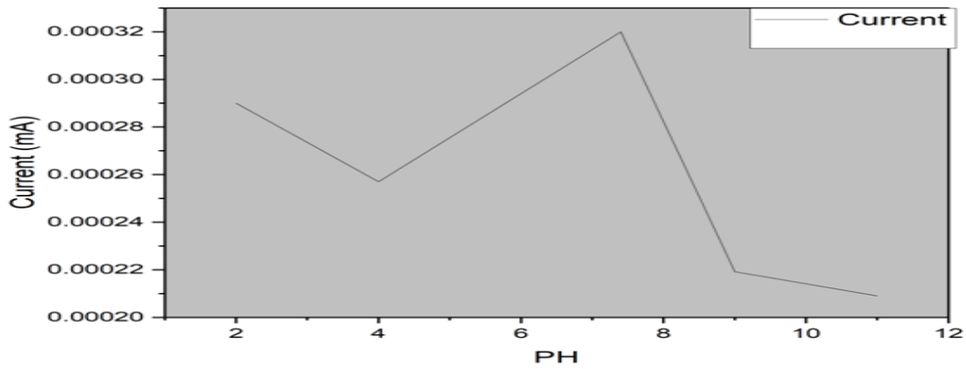

Figure 11: pH has been optimized for template extraction, created by Origin data analysis.

pH impacts the rebinding process, structure, and electron transfer rate of imprinted polymer sensing. Four separate MIPs were created and stored in 0.01 mM MA in buffer solution at various pH levels to examine the influence of pH on rebinding. The Highest peak current and rebinding happened at a pH of 7.4.

The target removal time, which varies from trial to trial and can range from a few minutes to many hours, determines the sensor's competitiveness. The imprinting technique and template removal create a milieu where MEL molecules might be found by their size, shape, and functional group location.

For the extraction of melamine from the MIP, some research studies advocate using a 1 M sulfuric acid solvent. The working electrode was immersed in H_2SO_4 to remove it from the coating. In a sulfuric acid solution at various time intervals, they produced binding holes like the target shape, size, and functionality. The elimination of MEL and the creation of template memory as evidenced by the present reaction remaining unchanged after 40 minutes (Figure 12). The template may be extracted from the MIP sensor after 40 minutes of washing.

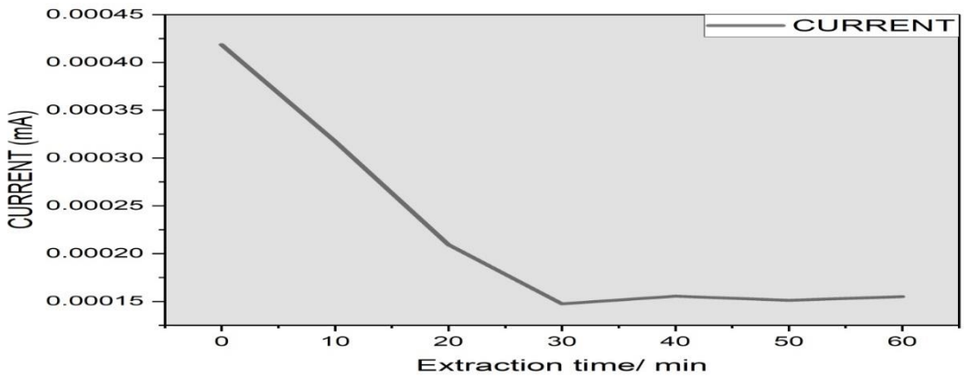

Figure 12: Time for template extraction and optimization, created by Origin data analysis

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MIP/GCE was incubated in PBS with 0.1µm MEL for 5 to 25 minutes in static mode for recognition research, which looked at how the film rebinds to the template. This was done to keep the sensor and MEL molecule in stable, non-covalent contacts for the best rebinding results. Based on the current finding, The rebinding period of ten min produced the maximum peak current and was selected as the ideal time for rebinding the MEL on the MIP films (Figure 12). The template has reached saturation and adsorbed calibration at this point.

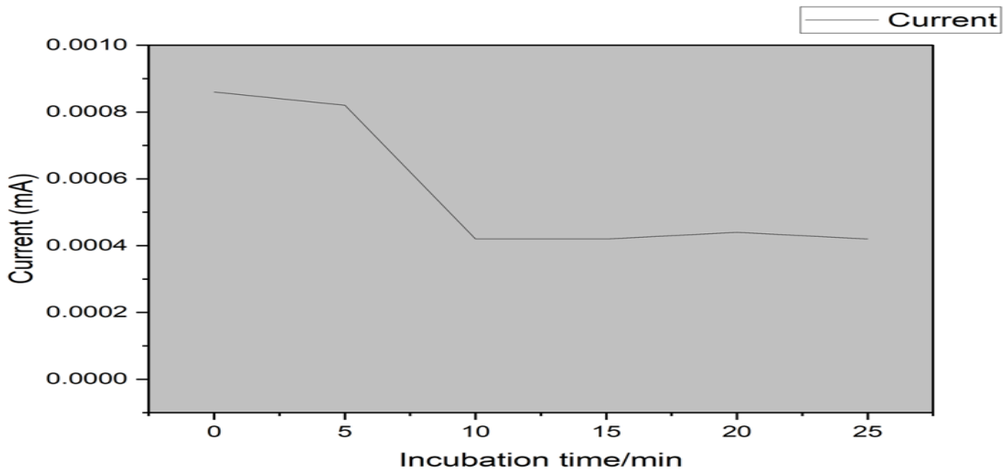

Figure 13: Optimization of incubation time/minute, created by Origin data analysis.

3.4 Detection of melamine using DPV sensor, the limit of detection, quantification, linearity, and affinity

Barker and Jenkin [71] were the first to introduce differential pulse Voltammetry. Their goal was to improve voltammetric measures' detection limitations. These techniques make it simple to measure down to the (10 to 8M) level to increase the ratio between Faradaic and non-Faradaic currents of functions redox species on the chemically modified electrode, and a DPV is superior. Because of the baseline current that might hide the Faradaic wind of connected redox molecules, the CV approach is less sensitive than the DPV method. Because of this, the CV method is less delicate when measuring subsurface amounts are modest.

In all the experiments, the DPV method was applied. The following circumstances were used in the experiments: the potential was recorded between 0.0 and +0.6 V, and the step voltage and modulated intensity were adjusted to the equipment default. The redox potential of the redox indicator in electrochemical MIPs was determined by comparing its redox potential to that of an Ag/AgCl reference electrode. With its increased current sensibility and higher resolution, DVP was used to estimate MEL. DPV was employed to see how the electrocatalytic activity of the electrode in PBS with varying concentrations of MEL affected current flow in the optimal circumstances for an investigation.

MEL content could be connected using a linear model. The calibration curve displayed as current versus the potential for various concentrations of MEL 0.1 to 5µM and in the PBS. From 0.1 to 1µM, the maximum responsivity and the negative logarithmic value of MEL.

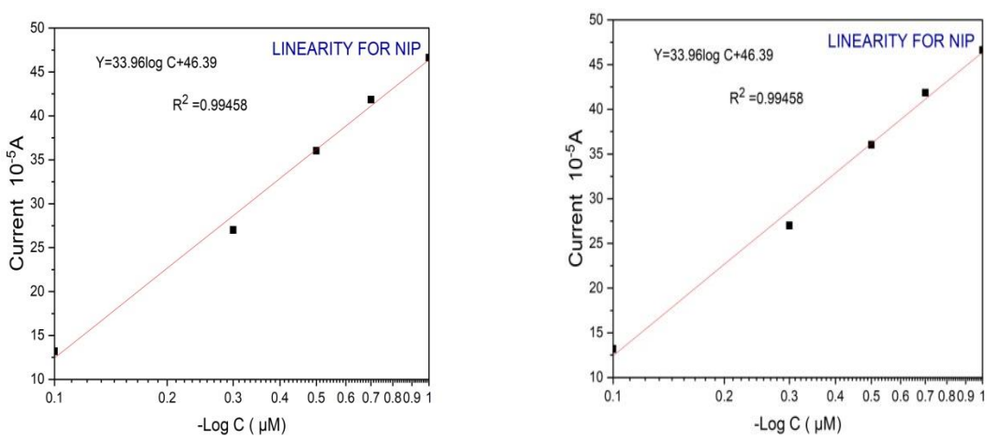

Figure 14: The calibration curve for melamine detection using the NIP/MIP sensor demonstrates linearity for the concentrations created by Origin data analysis.

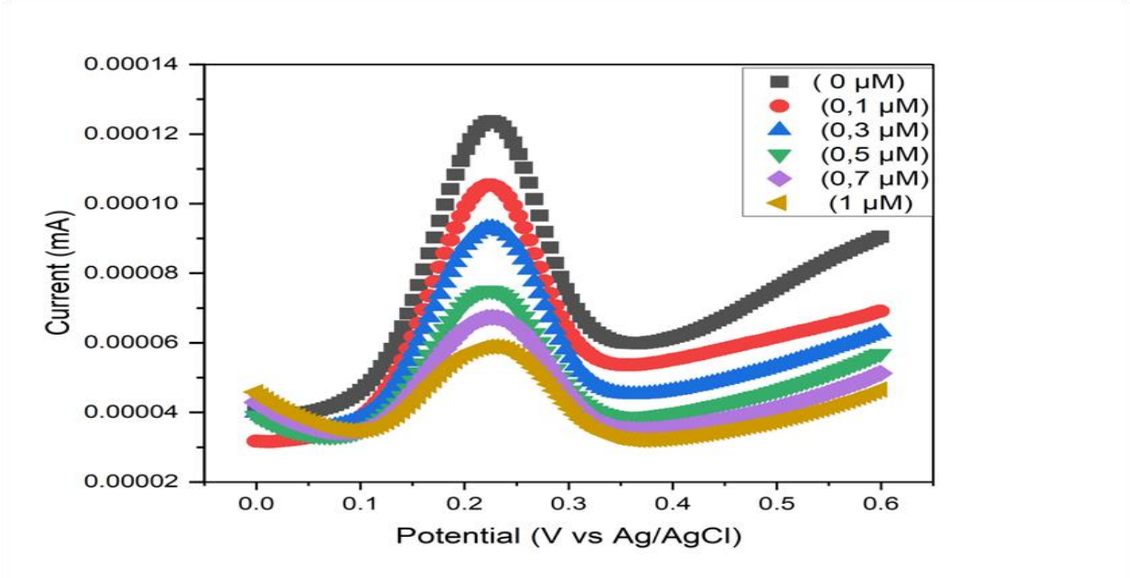

Figure 15: The DPV response was used to detect different amounts of melamine for linearity, created by Origin data analysis.

The current intensity increases as the amount of MEL is increased, indicating that the template uptake on the electroactive response is improved. The linear regression equation is I(mA) = 35.3logC+56.53(-log C)(µM), with r = 0.9957 as the correlation coefficient, where C is the MEL concentration. The computed LOD and LOQ for the MIP sensor are 90 and 280nM, respectively. While the NIP has shown the regression equation, I(mA) = 33.96logC+46.39(-log C)(µM), with r = 0.99277.

To assess how AuNP influences surface area and sensitivity. The addition of 6nM gold nanoparticles to both NIP and MIP increases the sensitivity of the working electrode, increasing surface area and boosting electric conductivity. One µM of the sample was used to analyze NIP and MIP, and relative sensitivity was demonstrated by increasing the current response, as seen below.

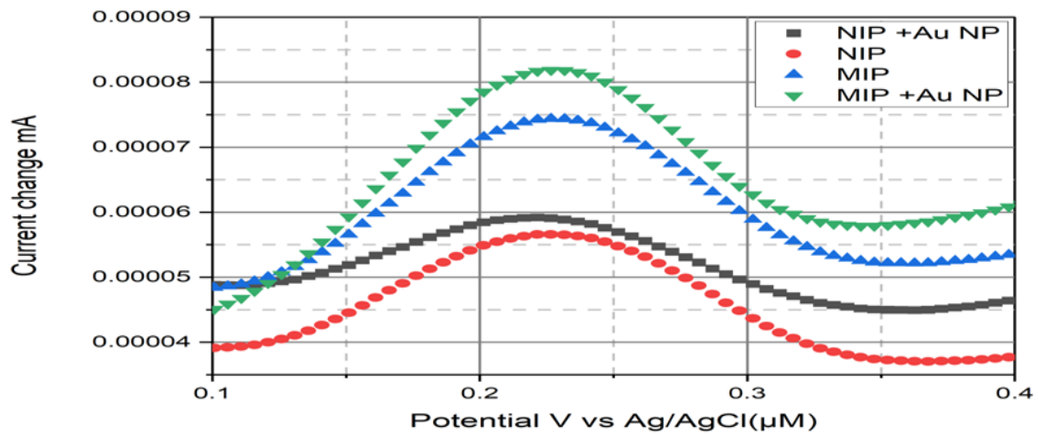

Figure 16: The effect of AuNP on the NIP/MIP sensor, created by Origin data analysis.

3.4.1 Binding affinity

The imprinting factor [72] offered good precision for figuring out MEL. The preceding formula was used to calculate MIPs/rebinding affinity electrode to melamine:

IF = ΔIMIP/ ΔINIP

where ΔIMIP is the measured current shift for non-imprinted Polypyrrole electrodes with much the same concentration of MEL, and ΔINIP is the present change acquired with molecularly imprinted Poly pyrrole electrodes with One and a half µM amount of MEL. The fact that the

value of IF is significantly higher than 1.2[73] based on results for determining the different quantities of MEL indicates that the material has a strong affinity for the motif.

3.5 Specificity and selectivity of melamine using DPV sensor

MIP sensor should be unique to the imprinted molecule after incubation. As a result, the MIP sensor's affinity for MEL is investigated and compared to GCE replies without NIP as a baseline (see Figure 17 below). According to the selectivity analysis of CV, the Imprinting effect is stronger than the influence of the electrode that has not been modified or imprinted.

Interfering analogous compounds' present responses, such as Caffeine, Xanthine, and theobromine, were employed to put the suggested sensor device's selection to the test. Incubation of the sensing element in 0.1M PBS with 0.01mM MEL, 0.01mM caffeine, 0.01mM theobromine, and 0.01mM Xanthine, respectively. According to the current data, caffeine, THBr, and ZAN had no discernible interfering impact on the sensor's identification of MA (Figure shown by DPV in comparison). This shows that the designed sensor can detect the template with high selectivity.

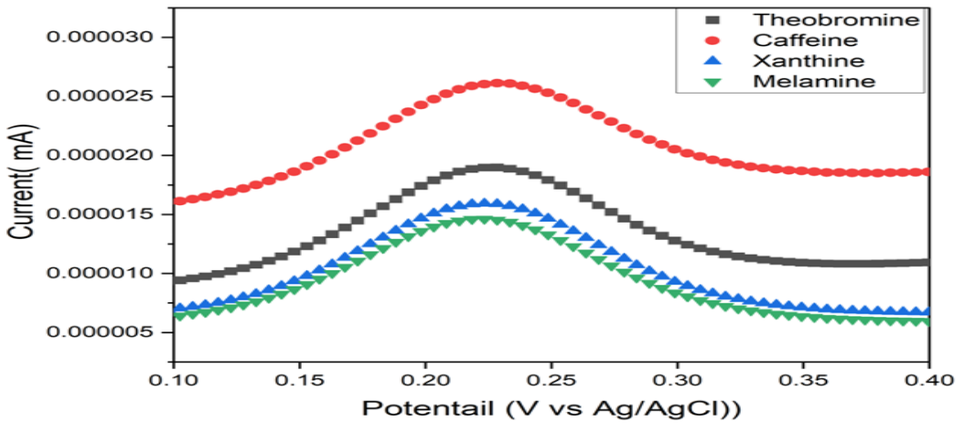

Figure17: Specificity study with the counterpart of melamine such as Caffeine, Theobromine, and Xanthine organic compounds, created by Origin data analysis.

3.6 Reproducibility of the sensor

The procedure and performance evaluation must be repeated; therefore, sensor production operations and tests must be repeatable. Three separate modified electrodes were manufactured independently using the same process to test fabrication reproducibility. At the intermediate 95 percent confidence level (0.05), the current response of the sensors after incubation in 0.1 µM MEL exhibited no significant variation. The developed procedure's precision in RSD (Relative Standard Deviation) was 3.7 percent, showing that the sensor's fabrication reproducibility is good. The ability of the MIP/GCE sensor for repeat measurements was also confirmed by taking three consecutive measures with the same measured quantity. The current response levels are 0.10674, 0.1000, and 0.10005 mA, with an RSD of 3.7 percent. Precision was utilized to assess the repeatability of the MIP sensor or how well-repeated observations coincide with each other, which is usually expressed as a standard deviation. The MIP/GCE sensor described is precise and can be used to locate MEL in several matrices.

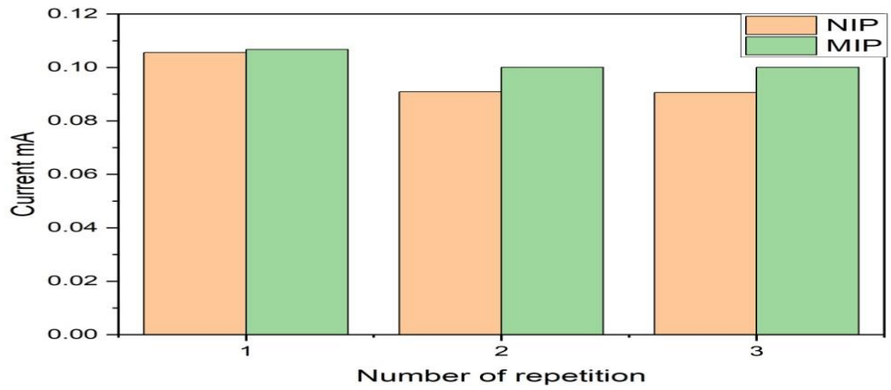

Figure 18: Reproducibility (Repeatability) measurement NIP/MIP Sensor, created by Origin data analysis.

3.7 Reusability and stability

The sensor's reusability has an intriguing characteristic. Without changing the extraction and incubation techniques to test the reusability and stability of the modified surface of the binding pockets, the strength and reusability of the NIP/MIP sensor were investigated using the same protocols as for template extraction and rebinding in the PBS solution.

The stability of the NIP/MIP sensor on different solvents was not tested due to time constraints. A few measurements were conducted, and the relative standard deviation was not within the acceptable range. After seven repetitions of extraction and incubation, it is around 7.3 percent, indicating excessive oxidation and saturation of the cavities. The pyrrole has swelling qualities that prevent the pockets from sensing increased oxidation.

Table 01: Compared published data to melamine detection methods based on MIP in this study

Technique	Limit of detection	Percent Recovery	Reference
EMIP sensor	175 pm	95	[74]
EMIP sensor	30.9nM	91	[75]
EMIP sensor	1.6 µM	95	[76]
MS/GC/MIP	6.9 mM	89.1	[77]
HPLC/UV	4.2 mM	91.1	[78]
MIP- Optical sensor	12 µM	-	[79]
EMIP sensor	5.3 µM	Less than 90	Present work

3.8 Authentic Melamine Sample Analysis

Application of the developed and the proposed sensor for the detection of melamine in an accurate liquid milk sample bought from the local store. The quantified melamine content was spiked in three different models and extracted using (chloroform: water), liquid-liquid extraction solvents, and then centrifuged for 10 minutes and filtered with a hydrophobic filter of 0.45 Micrometer at the end of the analysis; DPV measured the sample.

Table 02: Detection of melamine in liquid milk

Spiked amount (µg)	Detected amount (µg)	% Recovery
0.1	0.082	82
0.5	0.398	79.66
1.0	0.877	87.77

All the percentage recovery findings are out of the ordinary. This demonstrates that the matrix in the liquid milk influenced the analyte.

CHAPTER 4: General Conclusion and Future Work

4.1 Conclusion

MIPs with an sufficient affinity for melamine determination was prepared using conducting Poly pyrrole as the active motif for immobilization and signal enhancement. Small-sized functional thin films were formed by electropolymerization of working Poly pyrrole. The sensor fabrication molecular imprint is composed of weak bond interactions between the target and non-imprinted polymer and imprinted polymer. Using 1M Sulfuric acid solvent, the target was extracted from the MIPs, leaving pockets for rebinding the target. The selective and specific interaction of the target with MIPs makes the sensor for wide applications, including melamine determination.

The sensor can be regenerated with a short wash cycle in PBS and re-used in the right circumstances. MIPs have better stability than biological receptors, including antibodies and proteins, enabling the construction of durable devices. Despite its simplicity, electrochemical sensor applications require a better understanding of nanostructured materials that could be used for better utilization and excellent sensitivity. This electrochemical sensor was used in a real-world analysis of melamine that revealed a need for further optimization and sample purification before using it for research.

The sensor was designed specifically for the usage in an aquatic environment with good reproducibility and stability with a covariance coefficient of 3.75% but is also highly selective compared to melamine–like analysts like caffeine, Xanthine, and Theobromine. This developed electrochemical sensor based on MIPs for melamine determination showed excellent sensitivity within 0.1-1.0 μM. Least square line and I (MA) = 35.3 logC+56.53(-log C)(μM), Where C, Melamine concentration and r = 0.9957 is the correlation coefficient. The computed LOD and LOQ for the MIP Sensor are 90 and 280 nM, respectively.

3.2 Outline and future developments

Food security is crucial for people and animals looking to eradicate chemical contamination. Traditional analytical instruments are robust in detecting and sensing in response but lack selectivity, time, and solvent consumption and require experts. The benefit of molecular imprinting for melamine in conducting polymers is discussed in this thesis.

In this thesis work, electrochemistry has proved its role in the electropolymerization of pyrrole in small-sized thin films deposited to enhance the conductivity or functionality. Considering the morphological and MIP concepts useful in biomedical sciences, these MIPs could scale the commercial potential for more comprehensive applications, including biomarkers in oncogenic precision medicine, home-based analysis for multiple detections, and environmental monitoring for bacterial and viral waste, water treatment, and purification. For instance, in Chapter Two, many ideas need to be exploited. Many experimental tests have been left, including further optimization, stability tests, computational analysis, and mathematical modeling. It might be interesting to dope the conducting polymers. Furthermore, nanostructured materials in MIPs improve sensitivity and film quality. Film thickness should be assessed using an FT-IR spectrometer.

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