IMMOBILIZATION OF GLUCOSE OXIDASE AND POLYPHENOL OXIDASE IN POLY(N-(4-(3-THIENYL METHYLENE)-OXYCARBONYLPHENYL) MALEIMIDE)-CO-PYRROLE) MATRICE

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MAHMUT ÇİL

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FOR
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Approval of the Graduate School of Natural and Applied Sciences.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Sciences.

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ABSTRACT

IMMOBILIZATION OF GLUCOSE OXIDASE AND POLYPHENOL OXIDASE IN POLY(N-(4-(3-THIENYL METHYLENE)-OXYCARBONYLPHENYL) MALEIMIDE)-CO-PYRROLE) MATRICE

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In this study, glucose oxidase and polyphenol oxidase were immobilized in conducting copolymer poly(N-(4-(3-thienyl methylene)-oxycarbonylphenyl)maleimide)-co-pyrrole(P(MBThi-co-Py)). A copolymer was electrochemically synthesized by using sodium dodecyl sulfate (SDS) as supporting electrolyte and characterized by FTIR, scanning electron microscopy (SEM) and conductivity measurements.

Immobilization of glucose oxidase (GOD) and polyphenol oxidase (PPO) enzymes were performed in conducting PPy and P(MBThi-co-Py) matrices by electropolymerization. Kinetic parameters, maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) were determined for the enzyme electrodes by help of Lineweaver-Burk plot. Effect of temperature and pH on GOD and PPO activity was examined. Operational stability and

long term stability of the enzyme electrodes were investigated. The immobilized GOD and PPO electrodes were used for determination of glucose amount in Turkish orange juices and analyzing the concentration of phenolic compounds in Turkish red wines respectively.

Keywords: Electrochemical polymerization, enzyme immobilization, glucose oxidase, polyphenol oxidase, orange juice, wine.

ÖZ

GLUKOZ OKSİDAZ VE POLİFENOL OKSİDAZ ENZİMLERİNİN POLİ(N-(4-(3-TİYENİL METİLEN)-OKSİKARBONİLFENİL) MALEİMİD)-PİROL KOPOLİMER MATRİSİNDE TUTUKLANMASI

Çil, Mahmut
Yüksek Lisans, Kimya Bölümü
Tez Yöneticisi: Prof. Dr. Levent Toppare

Temmuz 2006, 65 sayfa

Bu çalışmada glukoz oksidaz ve polifenol oksidaz iletken kopolimer poli(N-(4-(3- tiyenil metilen)-oksikarbonilfenil)maleimid)-pirol) (P(MBThi-co-Py)) matrisinde tutuklandı. Kopolimer, sodyum dodesil sülfat destek elektroliti kullanarak elekrokimyasal olarak sentezlendi ve FTIR, taramalı elektron mikroskopi ve iletkenlik ölçümü yoluyla karakterize edildi.

Glukoz oksidaz ve polifenol oksidaz enzimleri iletken PPy and P(MBThi-co-Py) matrislerinde elekropolimerleşme yoluyla tutuklandı. Enzim elektrotlarının kinetik parametreleri, maksimum tepkime hızı (V_{max}) ve Michaelis-Menten sabitleri (K_m), Lineweaver-Burk grafiği yardımıyla belirlendi. Sıcaklık ve pH, glukoz oksidaz ve polifenol oksidaz aktiviteleri üzerindeki etkisi incelendi. Enzim elekrotlarının kullanım kararlılıkları

belirlendi. Glukoz oksidaz enzim elekrodu, portakal meyve suyunda glukoz miktarı tayinde ve polifenol oksidaz enzim elekrodu, kırmızı şarapta fenolik yapıların miktarını belirlemede kullanıldı.

Anahtar Kelimeler: Elektrokimyasal polimerleşme, enzim tutuklaması, glukoz oksidaz, polifenol oksidaz, portakal suyu, şarap.

To My Family

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ABREVIATIONS

BSA	Bovine serum albumin
CB	Conduction Band
E.C	Enzyme Commission
FAD	Flavin adenine dinucleotide
FTIR	Fourier infrared spectrometry
GOD	Glucose oxidase
MBTH	3-methyl-2-benzothiozolinone hydrazone
MBThi	N-(4-(3-Thienyl methylene)-oxycarbonylphenyl)maleimide
P(MBThi-co-Py)	Poly(N-(4-(3-Thienyl methylene)-oxycarbonylphenyl)maleimide-co-pyrrole)
POD	Peroxidase
PPO	Polyphenol oxidase
PPy	Polypyrrole
Py	Pyrrole
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
VB	Valence Band

CHAPTER I

INTRODUCTION

1.1 Conducting Polymers

Unlike metals, most polymers are insulating materials. Due to this property, they are used for many applications such as coating of electrical wires, as capacitor films etc. However, by the synthesis of the conducting polyacetylene in 1977, the study on the conductive polymers has gained popularity among the scientists. Conducting polymers are conjugated organic materials. These materials are different than the redox polymers since their backbone is intrinsically conducting. The conductivity of these materials can be improved by the insertion of dopants. The magnitude of the conductivity depends on the concentrations of dopants. The conductivity can be controlled in the range from insulator to that of metals. Figure 1.1 gives the chemical structure of some important conducting polymers.

1.1.1 History of Conducting Polymers

The first conducting polymer was polyaniline synthesized by the oxidation of aniline in sulphric acid by H. Letheby in 1862 [1]. In 1958, Natta and co-workers achieved to polymerize acetylene with the use of Ziegler-type

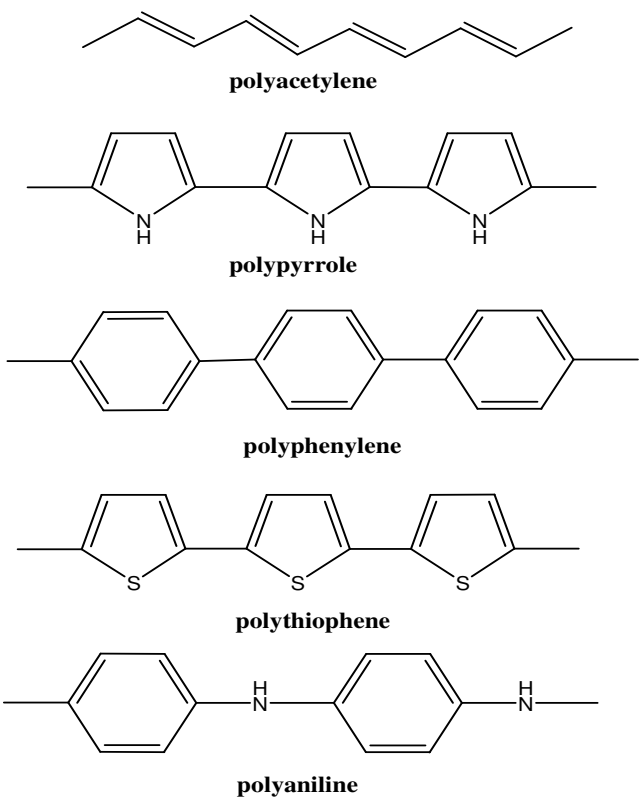


Figure 1.1 Some important conducting polymers

catalyst [2] and Ziegler and Natta were awarded with the Nobel Prize in Chemistry 1966. In spite of its highly crystalline and regular structure, it was a black, an air-sensitive, infusible and insoluble powder. Therefore, it was not used effectively until 1970s.

In the early 1970s, Shirakawa and co-workers synthesized a film of polyacetylene [3]. They synthesized this film by blowing the acetylene gas onto the surface of a catalyst solution. They found that conductivity of trans-polyacetylene was 10^{-3}- 10^{-2} s m^{-1} on the other hand, that of cis-polyacetylene was 10^{-8}-10^{-7} s m^{-1}. Also, they claimed that the cis form can be converted to trans form by heating Ca.170-200^{o}C.

In 1975, Alan Heeger and Alan MacDiarmid studied the metallic properties of a covalent inorganic polymer $(SN)_x$. Then, they focused on the synthesis of highly conductive polyacetylene. They worked with Shirakawa and discovered that the oxidation with chlorine, bromine and iodine vapors made polyacetylene film 10^9 times more conducting than the original one [4]. This high conductivity opened the field of 'plastic electronics'. They won the Nobel Prize in Chemistry 2000 for the discovery and development of electrically conducting polymers.

In 1980s, studies were done on the other polymers such as polypyrrole [5], polythiophene [6], and polyaniline [7] and their derivatives. Today, the studies on conducting polymers are being continued to improve their several properties. They can be used as electrodes in transistors, light emitting diodes, electrochromic devices.

1.1.2 Conduction Mechanism of Conducting Polymers

The electrical properties of the materials can be determined by their electronic structure, the band theory explains the electronic structure of materials reasonably. In the solid state, orbitals of each atom overlap with those of neighboring atoms in all directions to create molecular orbitals that are similar to that of small molecules. As these orbitals come together, they form energy bands. In figure 1.2, the highest occupied band is called as the valence band whereas lowest unoccupied band is called as the conduction band. The energy difference between two bands is called as the energy gap. For a

conductor, the energy gap does not exist hence, the electron promotes easily into the conduction band. On the other hand, for an insulator, the energy gap between two bands is large that they do not conduct electricity. Semiconductors have narrow band gaps and limited conduction occurs.

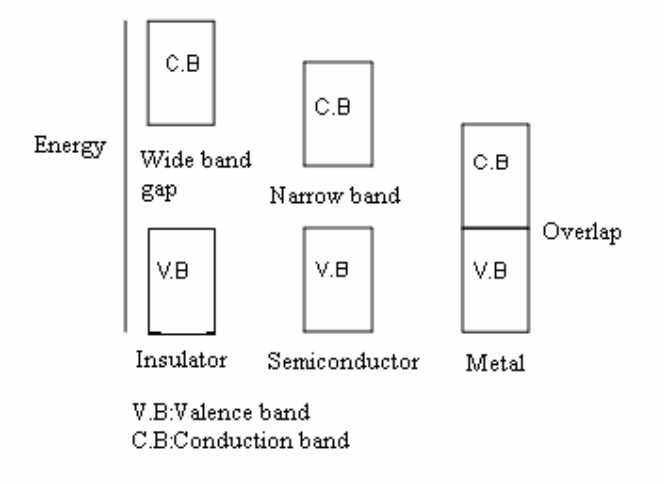


Figure 1.2 Simple band pictures of an insulator, a semiconductor, and a metal.

The electrical conduction of conducting polymers can not be given completely by the simple band theory since this theory can not explain the reason why charge carriers are spinless in polyacetylene and polypyrrole. To understand the electronic behavior of conducting polymers, the concepts of solitons, polarons, and bipolarons were introduced since 1980s [8].

Conducting polymers possess polyconjugated structures. They are insulators in the pristine state. They become conducting when treated with oxidizing or reducing agents. Thus, their conductivities can be increased by doping process. Doping is the addition of a donor or an acceptor molecule to the polymer. Doping is classified as p-type (oxidation) and n-type (reduction). P-type doping is the removal of electrons from the valence band by the oxidizing

agent, leaving the polymer with a positive charge. Unlike the p-type doping, n-type doping is the donation of an electron to the conduction band by a reducing agent.

The first step of the doping is the formation of a cation or an anion radical called a polaron. Then, second electron transfer takes place to form a dication or a dianion called as the bipolaron. Low doping levels give rise to polarons whereas the higher doping level produces bipolarons. In the electric field, polarons and bipolarons can move along the polymer chains by the rearrangement of double and single bonds. The energies of bipolarons can overlap and narrow bipolaron bands in the band gap can be formed by increasing in doping level as shown in figure 1.3. The highly conducting polymer is then obtained at a high doping level. The structures of a polaron and bipolaron for polypyrrole are shown in the figure 1.4**.**

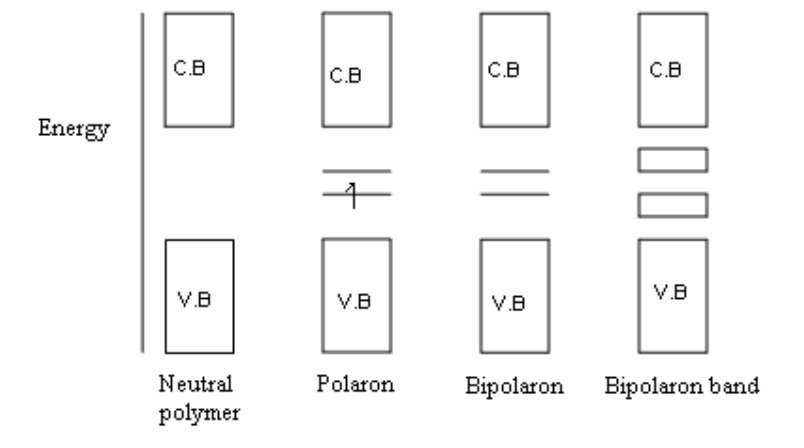


Figure 1.3 Band structures of neutral and doped polymer.

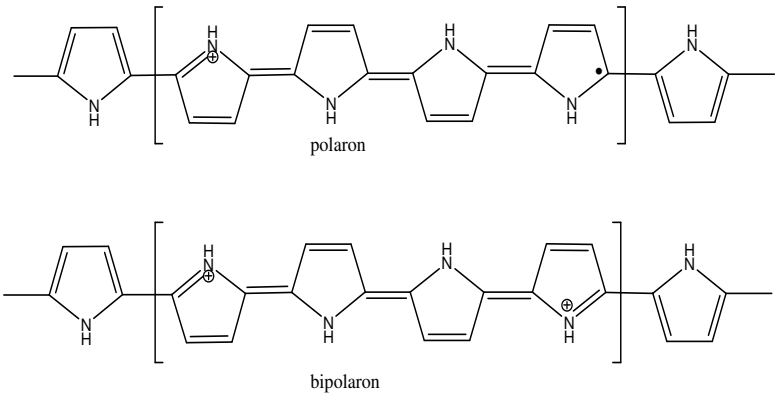


Figure 1.4 Structural schemes of polaron and bipolaron for polypyrrole

1.1.3 Electrochemical Polymerization of Conducting Polymers

Conducting polymers can be synthesized by chemical or electrochemical polymerization. They can be obtained by electropolymerization since most conjugated monomers can be oxidized electrochemically. In this method, polymerization starts when active species are formed on the electrodes. Electrochemical polymerization is carried out in a reaction medium of suitable conductivity and the polymers usually precipitate as a film on the electrode surface [9]. The oxidation potential of conducting polymer is lower than that of the monomer; therefore the polymer is simultaneously oxidized into conducting regine and kept electrically neutral by incorporation of the anion of the electrolyte.

For the electropolymerization of pyrrole, electrodes (platinum) were immersed in a solution containing the monomer, an aprotic solvent and a supporting electrolyte such as tetraethylammonium tetrafluoroborate [10, 11]. Electropolymerization starts with the oxidation of the pyrrole to yield a radical cation. Two radical cations come together to form a dication. Pyrrole dimer is formed after the elimination of two protons for rearomatization. The oxidation

potential of the dimer is lower than that of the monomer, hence polymerization continues by oxidative coupling of monomer units to the growing chain as shown in figure 1.5 [12]. As the molecular weight of growing chains increases, polymer becomes insoluble and deposits on the surface electrode where polymerization continues to give a high molecular weight polymer.

N-substituted pyrroles can be also polymerized. The conductivity of N-substituted polypyrrole is less than that of unsubstituted polymer. That is due to the fact that substitution prevents the coplanarity of the rings and disrupts effective conjugation [13, 14]

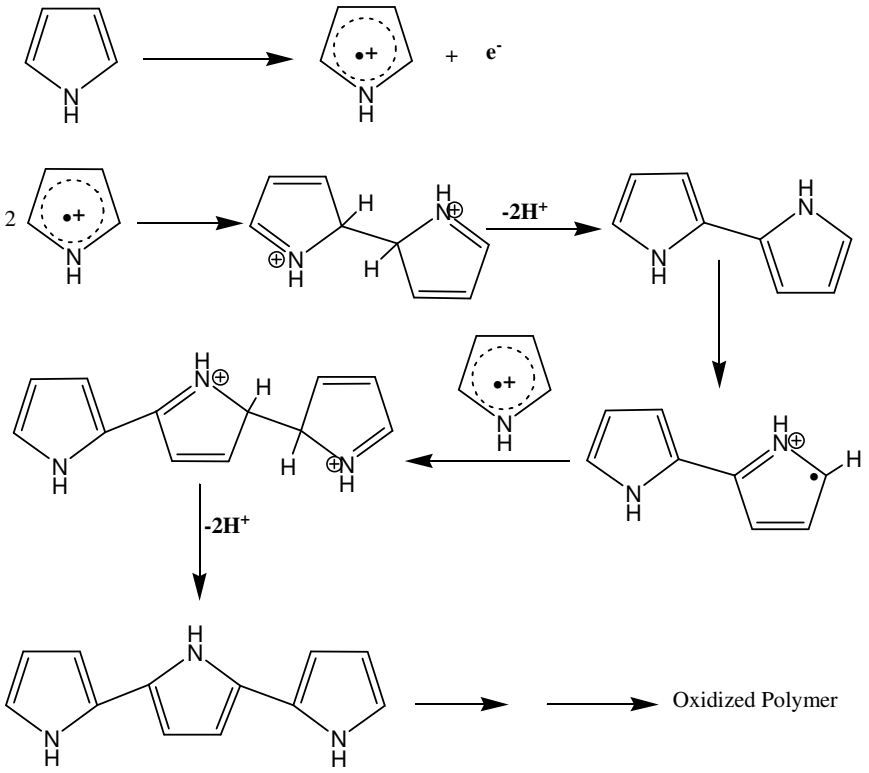


Figure 1.5 Electrochemical polymerization mechanisms for polypyrrole.

1.1.4 Applications of Conducting Polymers

The conductive polymers have been used for many applications since they have advantages of low density, low cost and ease of processibility. Some of applications are in batteries [15], capacitors [16], and electrochromic displays [17-19], field effect transistors [20, 21], light emitting diodes [22], photovoltaic [23], and photoelectrochemical cells [24-26]

1.2 Enzymes

Enzymes are catalysts that speed up the rates of reactions without themselves undergoing any permanent change [27]. They have been known for over a century. Swedish chemist Jon Jakob Berzelius performed the earliest studies in 1835 and found that the chemical action was catalytic. The first enzyme urease was isolated and crystallized from the jack bean by James B. Summer in 1926. He won the 1947 Nobel Prize. John H. Northrop and Wendell M. Stanley shared the 1947 Nobel Prize with Summer. They found the complex procedure for isolating pepsin. This procedure has also been used to crystallize several enzymes. Later, many studies were conducted on the enzymes. In 1986 Cech discovered that RNA can also act as a catalyst for reactions involving hydrolysis of RNA where rate enhancements of up to 10^{11} can be attained [28]. The RNA catalysts are known as ribozymes.

Substrates are substances enzymes act on. Many enzymes need the other component (cofactors) in order to catalyze the reaction. The presence of enzyme and cofactor are referred as the haloenzyme. Apoenzyme (protein portion) plus the cofactor (coenzyme) is called the holoenzyme. Most vitamins such as thiamin and niacin have a coenzyme in cells. Other coenzymes include nicotinamide adenine dinucleotide (NAD+), flavin adenine dinucleotide (FAD), and coenzyme A. The metal ions such as Cu^{+2}, Mg^{+2}, Zn^{+2} are bonded either to

the apoenzyme or to the coenzyme. The metal ions are usually designated as the enzyme cofactors.

Enzymes are highly specific substances which act on a specific substance or a specific type of substance and catalyze them for a particular reaction. Properties such as shape, hydrophilic and hydrophobic character and character of enzyme and substance are responsible for this specificity. For example, the enzyme invertase catalyzes sucrose to form simple sugars glucose and fructose. Hence, invertase acts on the substrate sucrose. The specificity can be explained by the Lock-Key model proposed by Emil Fischer in 1890. This model explains how an enzyme acts. The enzyme has an active site where substrate molecule binds and the reaction takes place. The substrate molecule (key) fits into the active site on the enzyme molecule (lock) to form an enzyme-substrate complex. Active site is important for specificity because it recognizes substrate and gives enzyme its specificity. While substrate connects the enzyme, the new bonds are formed to yield the products leaving the enzyme. The schematic diagram of Lock-Key model is shown in figure 1.6

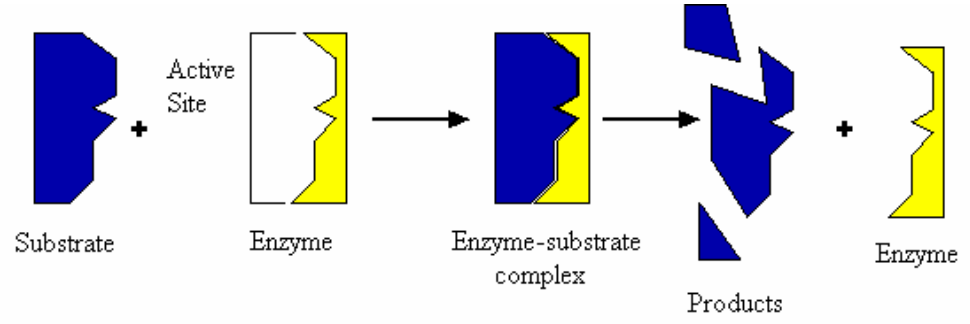


Figure1.6 Lock-Key model

Enzymes like all catalysts decrease the activation energy of the reaction and lead the reaction to proceed faster. They increase the rate of reaction about

10^{17}-fold. After completion of the reaction, they remain unchanged thus, they can be used many times. They do not affect the equilibrium of the reaction since they do not alter the relative energy between the products and reagents. Unlike other catalysts, the advantages of the enzymes are chemoselectivity and specificity.

The usage of the enzymes in food, pharmaceutical and chemical industries increase due to their catalytic properties. The enzymes are used in alcoholic beverages, breadmaking, cheesemaking, sweeteners, clarification of beers, wines, and fruitsjuices, and medical applications.

1.2.1 Enzyme Classification

International Union of Biochemistry established Enzyme Commission (E.C) for enzyme nomenclature due to the disappearance of harmony in naming the enzymes and increasing number of known enzymes. This commission suggested a systematic nomenclature (1984). E.C categorized all enzymes into six main groups according to the basis of reaction they catalyze. Each enzyme is donated with a numerical code compose of four units or numbers such as 3.5.1.5. The first number indicates the main group. Second and third numbers are related to subclasses and sub-subclasses. The last number describes the substrate that enzyme reacts with. Six main groups and type of reaction catalyzed are given below [29]

1) **Oxidoreductases**: Oxidation-reduction of all types.
2) **Transferases:** Transfer of an intact group of atoms from a donor to an acceptor molecule**.**
3) **Hydrolases**: Hydrolytic cleavage of bonds.
4) **Lyases:** Cleavage of C-C, C-O, C-N, and such other bonds, other than hydrolysis and oxidation includes reactions that eliminate water to leave double bonds or reacting where water is to a double bond.

5) **Isomereases:** Interconversion of various isomers such as cis-trans, L-D, aldehyde-ketone
6) **Lipases:** Bond formation due to the condensation of two different substances with energy provided by ATP.

1.2.2 Enzyme Kinetics

Kinetic features of enzyme-catalyzed reaction are important for gaining insight into enzyme behavior. The first study on the enzyme kinetics was done by V. Henri in 1903 and then, L.Michealis and Maud L. Menten in 1913.They studied on the same model however, the study of Michealis and Menten depends on experimental data. Their studies are believed to be the basis of enzyme kinetics. The kinetic mechanism of enzymes is explained for one-substrate by a simple model suggested by Michealis and Menten [30, 31]. The enzyme-catalyzed reaction of one-substrate mechanism is shown below.

$$\text{Enzyme(E)} + \text{Substrate(S)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Enzyme-Substrate Complex(ES)}$$

$$\text{Enzyme-Substrate Complex(ES)} \xrightarrow{k_3} \text{Enzyme(E)} + \text{Product(P)}$$

where k_1, k_2 and k_3 represent the rate constants.

Here are steps for the derivation for Michealis-Menten kinetic equation.

1) The rate of formation of ES is equal to that of the disappearance of ES. This is achieved very rapidly and called as steady-state equilibrium. The rate equations are:

Rate of ES formation $= k_1[E][S]$
Rate of ES disappearance $= k_2[ES] + k_3[ES]$

According to steady-state equilibrium,

$$k_1[E][S]= k_2[ES]+k_3[ES] \quad \text{.....1.1}$$

2) The total enzyme concentration $[E_t]$ is equal to the sum of the enzyme complex [ES] and free enzyme $[E_f]$.

$$[E_t]=[ES] + [E_f] \quad \text{.....1.2}$$

3) Initial velocity of the reaction according to rate of product formation by ES $\xrightarrow{k_3}$ E + P is given by

$$V_o=k_3[ES] \quad \text{.....1.3}$$

4) A maximum initial velocity (V_{max}) is achieved when all enzymes can form complex with substrate. When $[E_f]$ =0, $[ES]_{max}= [E_t]$ since $[E_t] = [ES] + [E_f]$. Hence V_{max} is directly proportional to $[E_t]$.

$$V_{max}= k_3[ES]_{max}= k_3[E_t] \quad \text{.....1.4}$$

By the combination of equations 1.1, 1.2, 1.3, 1.4, rate equation is obtained. From the equation 1.1, the equation is obtained for [ES] where [E] is $[E_f]$.

$$[ES]= (k_1/k_2 + k_3)[E_f][S] \quad \text{.....1.5}$$

$(k_1/k_2 + k_3)^{-1}$ are called as the Michealis-Menten constant (K_m). K_m is substituted into equation 1.5, and the equation becomes

$$[ES] = \frac{[E_f][S]}{K_m} \quad \text{.....1.6}$$

$[ES]=V_o/k_3$ from equation 1.3 is inserted into the equation 1.6

$$Vo = \frac{k_3[E_f][S]}{K_m} \quad \text{.....1.7}$$

$[E_f]= [E_t]-[ES]$ from the equation 1.2 is substituted for $[E_f]$ into equation 1.7 to obtain

$$Vo = \frac{(k_3[E_t][S]-k_3[ES][S])}{K_m} \quad \text{.....1.8}$$

$V_o=k_3[ES]$ and $V_{max}= k_3[E_t]$ from equations 1.3 and 1.4 respectively are substituted into equation 1.8 to get

$$Vo = \frac{V_{max}[S]-Vo[S]}{K_m} \quad \text{.....1.9}$$

To arrange this equation for V_o to obtains

$$Vo = \frac{V_{max}[S]}{K_m + [S]} \quad \text{.....1.10}$$

The last equation (1.10) is called the Michealis-Menten kinetic equation and graphical representation of the equation is shown in figure 1.7

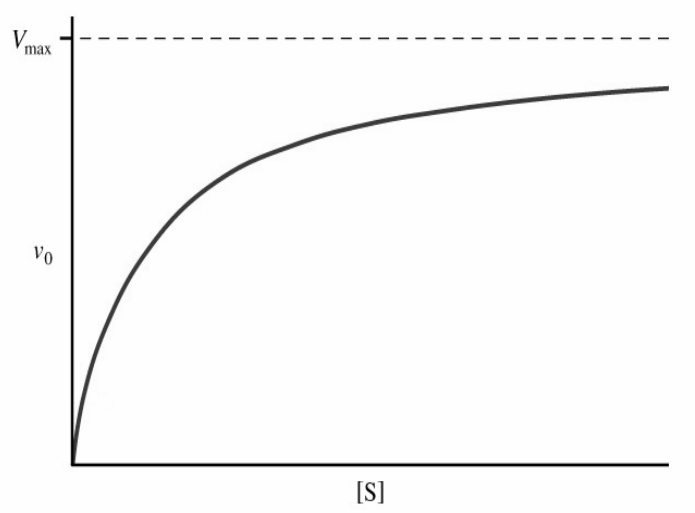


Figure 1.7 Graphical representation of Michealis-Menten kinetic equation

V_{max} and K_m are estimated directly from Michealis-Menten plot. V_{max} estimate is done from upper plateau region of the hyperbolic curve. K_m is equal to [S] when $Vo=V_{max}/2$. However, V_{max} and K_m are not evaluated exactly from Michealis-Menten plot because many enzymes give a saturation curve. To overcome this problem, Lineweaver and Burk [32] rearranged the Michealis-Menten equation to estimate kinetic parameters accurately. The rearrangement is as follows:

$$Vo = \frac{V_{max}[S]}{K_m + [S]} \text{ is inverted to } \frac{1}{Vo} = \frac{K_m + [S]}{V_{max}[S]}$$

$$\frac{1}{Vo} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad \text{.....1.11}$$

This equation (1.11) is the form of a simple straight line equation y=mx+n where y=1/Vo and x=1/[S]. If 1/Vo is plotted as function of 1/[S], y intercept is $1/V_{max}$, the slope is K_m/V_{max}, and x intercept is $-1/K_m$. As a result, K_m and V_{max} can be evaluated accurately using Lineweaver-Burk plot as shown in figure 1.8

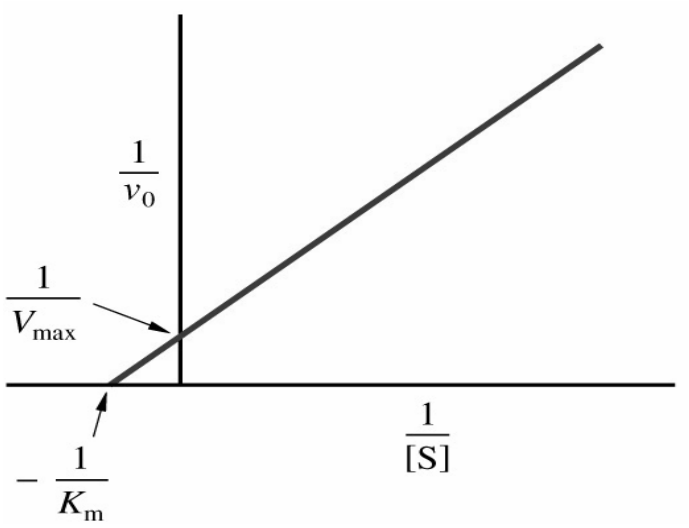


Figure 1.8 Lineweaver-Burk plot

1.2.3 Effect of Factors on Enzyme Activity

There are several factors that affect enzymes activity and reaction rate. These factors are temperature, pH, enzyme concentration, and substrate concentration

1.2.3.1 Effect of Substrate Concentration

Substrate concentration has an effect on the reaction rate of the enzyme. At constant enzyme concentration, if the substrate concentration is increased, the reaction rate increases until it reaches a maximum. After this point, rate does not increase with increasing substrate concentration since enzyme is saturated with the substrate. This is represented in figure 1.9.

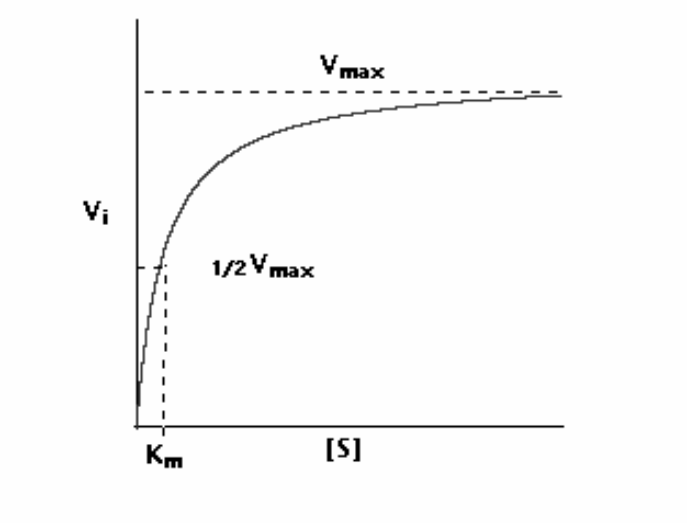


Figure 1.9 Effect of substrate concentration

1.2.3.2 Effect of Temperature

The rate of reaction increases as temperature is increased. This situation is complicated for the enzymatic reaction because of nature of the enzymes. Many enzymes are adversely affected by high temperatures. The reaction rate of enzyme increases with temperature up to a maximum temperature then, decreases suddenly with the increase in temperature as shown in figure1.10. This is due to the fact that most enzymes are rapidly denatured at temperatures

above 40·C, therefore most enzyme determinations are carried out somewhat below that temperature. Also, some enzymes lose their activity even below 0^{o}C.

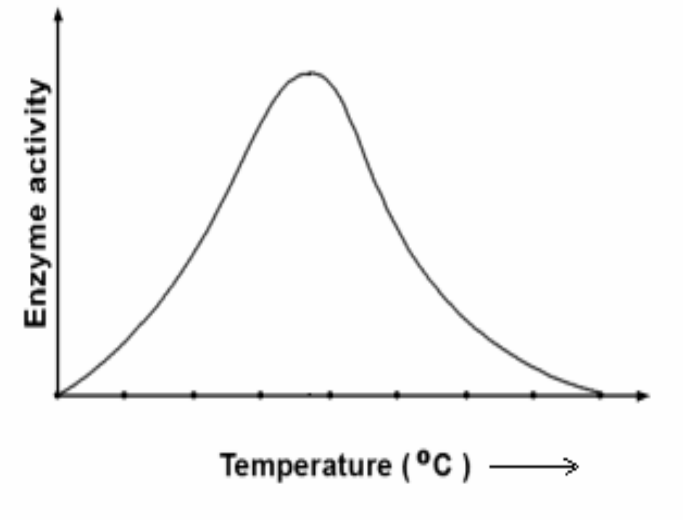


Figure 1.10 Effect of temperature

1.2.3.3 Effect of pH

Enzyme activity is also affected with changing pH. Effect of pH is shown in figure 1.11. Most enzymes lose their activities completely at extremely low or high pH values. Also each enzyme has an optimal pH stability region where they function effectively. The optimum pH value depends on the enzyme nature. Urease reveals maximum activity at pH 7.0 whereas invertase at pH 4.5.

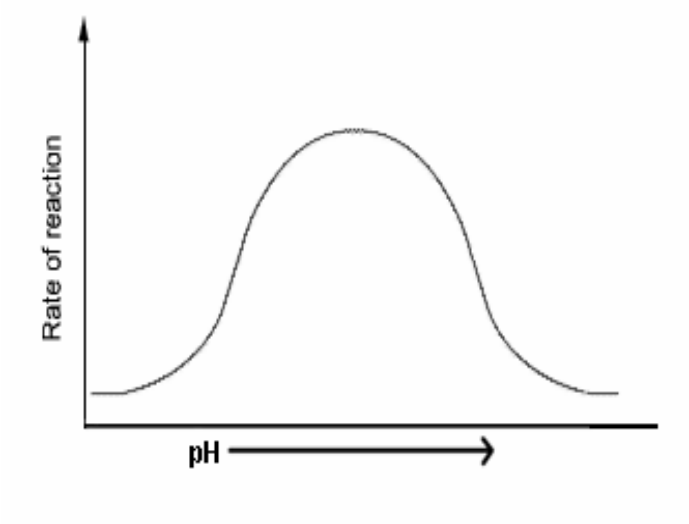


Figure 1.11 Effect of pH

1.3 Enzyme Immobilization

Immobilization is a way to enhance repetitive use of the enzymes. Immobilized enzyme was firstly mentioned at the Enzyme Engineering Conference in 1971. Immobilized enzyme is defined as the enzyme attached to an inert material without a loss in catalytic activity.

1.3.1 Benefits of Immobilization

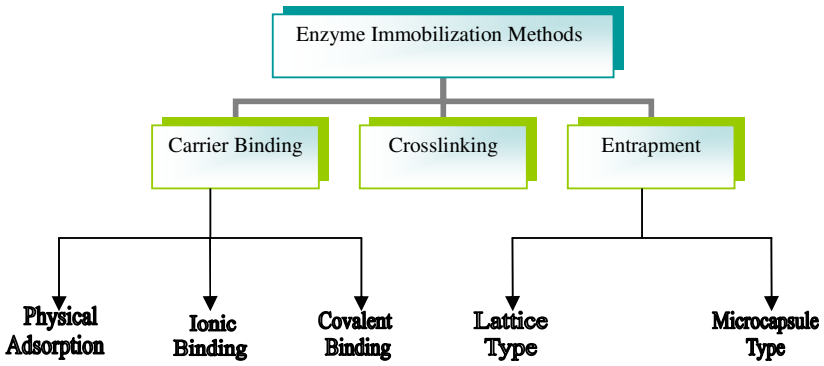
There are a number of benefits to attach enzymes into an inert material.

- Multiple use of a single batch of enzymes
- Reaction is stopped rapidly as enzyme is removed from the reaction solution or vice versa.
- Product is not contaminated with enzyme. This is beneficial for the food and pharmaceutical industries.

- Separation of enzyme from the product easily.
- Effluent disposable problems are reduced.

1.3.2 Methods of Enzyme Immobilization

The selection of the methods for the enzyme immobilization is important due to the activity of the enzyme. The active site of the enzyme should not be affected by the selected methods. The methods should protect the enzymes against environmental conditions such as temperature. Immobilization should not cause conformational changes and disturb the active site of enzymes. The best method is, the less it damages the enzyme. Types of immobilization methods are shown in the chart below:



1.3.2.1 Carrier Binding

This is the oldest method for immobilizing the enzymes. Enzyme is bound to water insoluble carriers. The amount of bound enzyme and activity of enzyme depend on the nature of carrier. The nature of the enzyme, such as particle size, surface area, molar ratio of hydrophilic to hydrophobic groups are

the parameters taken into account for the selection of the carrier. A higher immobilized enzymes activity is observed if the ratio of hydrophilic groups and the concentration of bound enzymes are high. Cellulose, polyacryamide are examples of carriers which are commonly used for the enzyme immobilization. This method is divided into three subgroups: physical adsorption, ionic binding and covalent binding.

- **Physical Adsorption:**

In this method, the enzyme protein is physically adsorbed on the surface of water insoluble carriers. During immobilization, little or no conformational change of enzyme occurs. This method is also simple and cheap. Disadvantage of this method is the leakage of the adsorbed enzyme from the carrier due to weak binding force between enzyme and carrier.

- **Ionic Binding:**

Ion-exchange residues such as polysaccharides and synthetic polymers are used as carriers. The enzyme is bound ionicaly to ion-exchange residues. The conformation and active site of the enzyme are affected very little thus, the enzyme reveals high activity. As is the case for physical adsorption, leakage of enzymes from the carrier takes place in this method.

- **Covalent Binding:**

This method is one of the most studied techniques for enzyme immobilization. During the immobilization, a covalent bond is formed between the enzyme and the support matrix. There are two limitations to select the type of reaction for the enzyme immobilization. The first is that the conditions used for the binding reaction should not cause loss of enzymatic activity. The second

is that the active site should not be affected by the reagents used. Unlike physical adsorption and ionic binding, covalent binding can alter the conformation and active center of the enzymes. As a result major loss of activity may occur.

1.3.2.2 Cross Linking

The enzyme immobilization is attained by formation of a covalent bond between enzyme molecules. The enzyme forms cross-linking with the other protein molecules or functional groups on insoluble support matrix. In this method, very little desorption occurs. It is also used for preventing leakage. On the other hand, it causes significant change of active site of the enzyme yielding some loss in the activity. Glutaraldehyde is most commonly used reagent for cross-linking.

1.3.2.3 Entrapment

This is the best method to immobilize the enzyme. The enzyme is immobilized into polymer matrix or membrane. During the immobilization, the enzyme is not interfered thus, the enzyme does not lose activity. This method is classified into lattice and microcapsule types. This method is extremely popular for whole cell immobilization in which usually natural polymers are employed due to their nontoxicity.

1.4 Glucose Oxidase

Glucose oxidase (E.C. 1.1.3.4.) is a flavoprotein which catalyzes the oxidation of β-D-glucose to δ-gluconolactone which is subsequently hydrolyzed to gluconic acid and hydrogen peroxide [33]

Glucose oxidase is found in many fungal sources such as aspergillus oryzae, penicillium notatum. Although glucose oxidase enzymes are purified from different sources, they share certain similarities in that all are dimers composed of two identical subunits, having 2 moles of firmly bound FAD per mole of protein, and containing 11-12 % carbohydrates [34]. Molecular weight of glucose oxidase purified from Aspergillus nigar is 160 kD having an optimum pH of 5.5 and isoelectric point of 4.2.

Glucose oxidase catalyzes the irreversible oxidation of mannose, xylose, and galactose. The reaction rates of these substrates are very low compared to that of D-glucose. This reality was confirmed by NMR studies [35]. If OH and CH_2OH groups on the substrate are at equatorial position, the reaction rate is significantly higher. On the other hand, if OH on C1 is replaced with H, enzyme loses its catalytic activity completely. Changes at C2 through C6 in the substrate reduce the catalytic efficiency of enzyme.

Glucose oxidase catalysis reaction consists of two half-reaction [36].

1) Reduction half-reaction in which E-FAD is reduced to E-$FADH_2$ and substrate is oxidized to the product.

2) Oxidative half-reaction in which E-$FADH_2$ is oxidized to E-FAD and the electron acceptor O_2 is reduced to H_2O_2 and also product is hydrolyzed to gluconic acid. Reaction diagram is shown in figure 1.12.

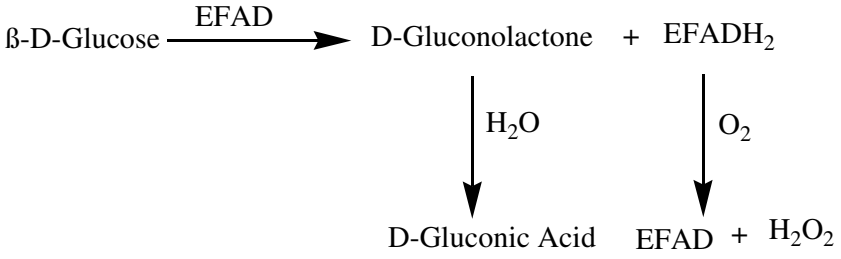


Figure 1.12 Reaction diagram for glucose oxidase

Glucose oxidase was immobilized on different supports with different immobilization methods [37-43]. Glucose oxidase has been used in many applications. Industrially it has been used to produce gluconic acid. It is also as a food preservative [44] and for the measurement of glucose in foodstuffs and beverages [45]. Its most important use has been in diagnostics such as in the determination of glucose in blood, serum or plasma [45].

In this work, glucose oxidase electrodes were utilized to determine the glucose amount in two kinds of Turkish orange juices, brand M and brand D.

1.5 Polyphenol Oxidase (Tyrosinase)

Polyphenol oxidase (PPO, EC. 1.14.18.1) otherwise known as tyrosinase was discovered by Bertnord and Bourquelot about 100 years ago. They recognized this enzyme when they were analyzing different types of mushrooms. They observed that mushrooms became brown dark at the end of reaction. Other studies indicated that tyrosinase catalyzed the aerobic oxidation of monophenols and the final product of tyrosinase oxidation was melanin [46].

Polyphenol oxidases are copper containing enzymes and have a coupled binuclear copper containing active site [47]. It catalyzes two different reactions using molecular oxygen. These reactions are the orthohydroxylation of monophenols to o-diphenols known as monophenolase activity and the oxidation of o-diphenols to respective quinones referred as diphenolase activity [48] (Figure 1.13). Quinones are very reactive compounds which undergo spontaneous polymerization reactions leading to the formation of brown pigments. Polyphenol oxidase is found in banana [49], potato [50], mushroom [50] avocado [50], and apple [51]. Tyrosinase is responsible for browing in plants and for melanization in animals. In animals, polyphenol oxidase provides the biosynthesis of melanins in skin, hair and eyes. These melanins play roles to protect the animal from solar radiation and predators.

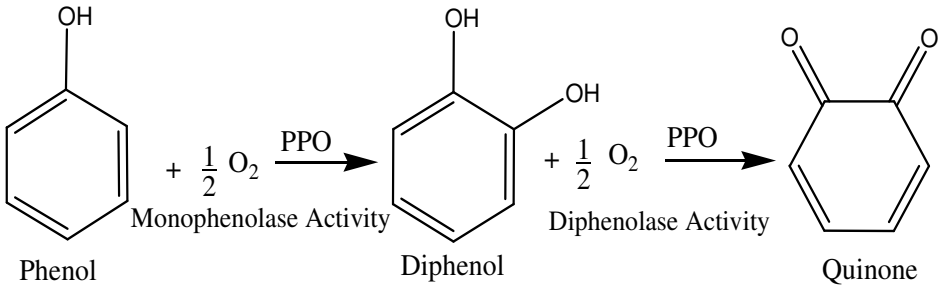


Figure 1.13 Enzyme activity mechanism of PPO

Inhibitors for polyphenols oxidase are important because they are used as antitumural agents against malignant melanoma [52] and as antibrowing agents in processing fruits and vegetables [53].

Immobilization of polyphenol oxidase in a matrix such as carbon paste [54], hydro-gel [55] and conducting polymers [56-58] have been reported in the literature. Polyphenol oxidase has been used for the determination of phenolic compounds in the wines, beers and wastewater. Especially, the studies were done to determine the phenolic compounds in the wines and there are several studies in the literature [59-61].

There are many biologically active compounds in red wines. Polyphenols are the most important ones due to their antioxidant properties. Flavonoids and related phenolics in the wine serve for the protective effect of red wine on the corononary heart diasase [62] and the oxidation of human low-density lipoproteins [63]. Therefore, to evaluate the antioxidant capacity of wines in relation to their phenolic constituents has gained interest among the scientists [64].

Phenolic compounds are responsible for color and contribute to the bitter flavor of wines. The composition and concentration of phenolic in wine depend on the type of grape used for vinification, the procedure employed for wine making and the chemical reactions taking place during the aging of the wine

[65]. In this study, polyphenol oxidase electrodes were used to determine concentration of phenolic in two kinds of Turkish red wines.

1.6 Aim of the Study

1- To synthesize and characterize the conducting copolymer of monomer (MBThi) with pyrrole by electropolymerization.

2- To test the copolymer for the immobilization of glucose oxidase and polyphenol oxidase.

3- To characterize the enzyme electrodes in terms of;

- Kinetic parameters
- Temperature and pH stabilities
- Operational and long-term stabilities

4- To test enzyme electrodes on the real samples

CHAPTER II

EXPERIMENTAL

2.1. Materials

Pyrrole(Merck) was distilled before use and stored at 4 °C. Dichloromethane (Merck), supporting electrolyte, sodium dodecyl sulfate (Sigma) were used as purchased. Sulfuric acid was obtained from Merck.

Polyphenol oxidase (PPO, E.C: 1.14.18.1, 1,530 units/mg solid), glucose oxidase (GOD, EC: 1.1.3.4, 47,200 units/g solid), peroxidase (POD, EC: 1.11.1.7), o-dianisidine, and substrates catechol and glucose were purchased from Sigma. Sodium acetate, citric acid, and 3-methyl-2-benzothiozolinone hydrazone (MBTH) were supplied from Sigma.

For the preparation of Lowry reagent, sodium carbonate (Delta), sodium potassium tartrate (Baker and Adamson), copper(II) sulfate pentahydrate (Merck), panreac sodium chloride(Montplet & Estaban SL), sodium hydroxide(Sigma) were used as received . Bovine Serum Albumin and Folin & Ciocalteu's Phenol Reagent from Sigma were used as purchased.

2.2 Experimental Methods

2.2.1 Electrolysis

In order to synthesize the conducting polymer and immobilize the enzymes in this conducting polymer, constant potential electrolysis was performed. Potentiostat was used to keep the potential difference between working and reference electrodes at a present value. It compensates the voltage drop in the electrolysis medium. This is accomplished by comparing continuously the potential difference between working electrode potential and reference electrode. In this study, Wenking POS-73 model potentiostat was used.

A typical three electrode cell was used to synthesize the copolymer and immobilize the enzymes. It consists of platinum electrodes as working, and counter electrodes, and a silver wire reference electrode. A typical three electrode cell is given in figure 2.1.

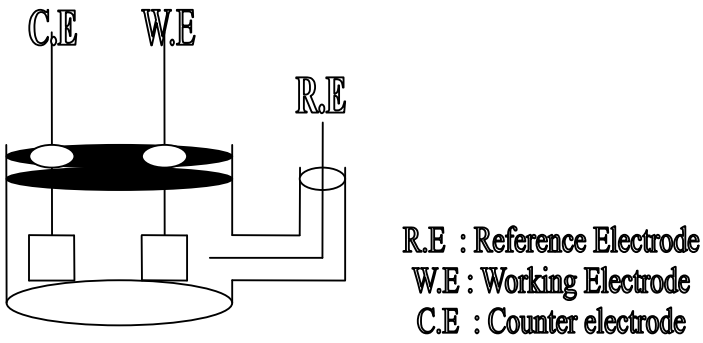


Figure 2.1 A typical three electrode cell

2.2.2 Four Probe Conductivity Measurements

Four probe technique was used to measure the conductivity of the conducting polymers.

2.2.3 Fourier Transform Infrared Spectrophotometry (FTIR)

Varian 1000 FTIR was used to obtain the FTIR spectra of monomer and conducting polymer.

2.2.4 Scanning Electron Microscope (SEM)

The surface morphologies of films were performed by a JEOL model JSM-6400 scanning electron microscope.

2.2.5 UV-Visible Spectrophotometer

A Shimadzu UV-1601 model spectrophotometer was used to determine the enzyme activity of both free and immobilized enzymes.

2.3 Experimental Procedures

2.3.1 Synthesis of MBThi and Copolymer of MBThi with Pyrrole

The synthesis and characterization of monomer (MBThi) (Figure 2.2) have been described by F.Yılmaz et al [66].

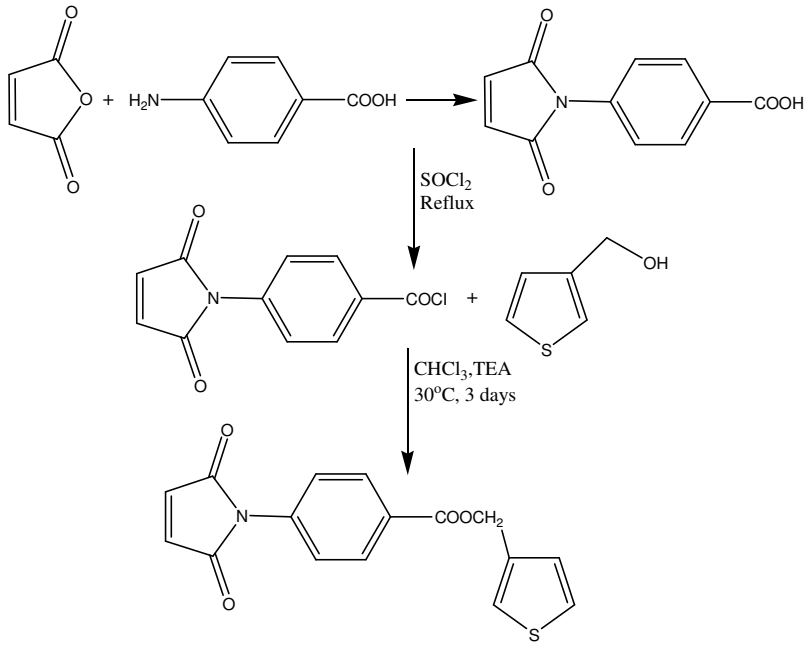


Figure 2.2 Synthesis of monomer (MBThi)

The mechanism of synthesis of random copolymer of MBThi with pyrrole was shown in figure 2.3. Working electrode was coated with dichloromethane solution of MBThi (1%w/v). After evaporation of solvent, the electrode was then immersed into cell containing 10ml H_2O, 50µl pyrrole and 0.006gr SDS. 30 min. of electropolymerization was preformed by applying +1.0 V potential against a silver wire reference electrode. The resulting copolymer was coded as P(MBThi-co-Py).

$$\text{Pt(MBThi coated)} \xrightarrow[\text{SDS, } H_2O]{\text{Pyrrole, + 1.0 V}} \text{P(MBThi-co-Py)}$$

Figure 2.3 Electrochemical route for copolymerization of MBThi with pyrrole.

2.3.2 Immobilization of Enzymes

2.3.2.1 Glucose Oxidase Immobilization

2.3.2.1.1 Immobilization Procedure of Glucose Oxidase (GOD) in Poly(MBThi-co-Py)

Entrapment of glucose oxidase was achieved by the electropolymerization of pyrrole either on bare or previously MBThi coated platinum electrode. A typical three electrode cell was used for the electropolymerization. 20mg (2mg/ml) glucose oxidase (47,200 units/g), 6mg (0.6mg/ml) SDS as supporting electrolyte, 50µl pyrrole, and 10 ml acetate buffer were mixed as the electrolysis medium. The platinum electrodes were used as the working and counter electrodes while a silver wire was the reference electrode. The working electrode was coated with MBThi monomer. Electropolymerization was performed by applying +1.0 V for 30 min. After electropolymerization, enzyme electrodes were washed in order to remove unbound enzyme and SDS and kept in acetate buffer when not in use.

2.3.2.1.2 Assay of Glucose Oxidase Activity

The principle and measuring the activity of free glucose oxidase is determination of the amount of the hydrogen peroxide formed. A modified version of Sigma Bulletin was used to measure the activity of glucose oxidase [67]. For free glucose oxidase, 0.1ml (2mg/ml) glucose oxidase were added to 2.9 ml glucose solutions prepared in acetate buffer (pH=5.1) for 1, 2, and 3 min at 25^{o}C. Then, 0.1ml peroxidase (60 U/ml) and 2.4 ml o-dianisidine (0.21mM) as the coloring agent were added to 0.5 ml solution drawn from the mixture. Finally, 0.5 ml H_2SO_4 (2.5 M) were added to stop the enzymatic reaction. The absorbance was measured at 530 nm.

For the determination of the immobilized glucose oxidase, the enzyme electrode was immersed in 3ml glucose solutions for 2, 4 and 6 min at 25°C. Then, enzyme electrode was removed and 1ml solution was drawn. The rest of the procedure is the same as that of free glucose oxidase. H_2O_2 standart curve was used to define enzyme activity. One unit of enzyme activity is expressed as the amount enzyme required to produce 1μmol of gluconic acid and H_2O_2 per minute at pH=5.1 and 25°C. Assay reactions for glucose oxidase are shown in the figure 2.4

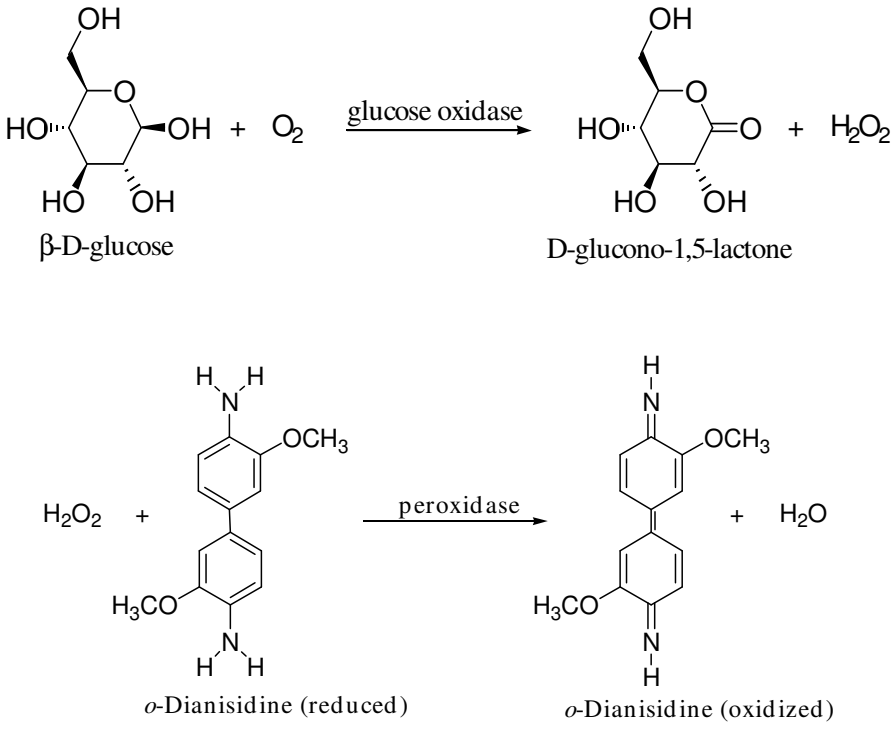


Figure 2.4 Assay reactions for glucose oxidase.

2.3.2.1.3 Determination of Kinetic Parameters of Immobilized Glucose Oxidase

Kinetic parameters (K_m, V_{max}) of free and immobilized GOD were investigated at constant temperature (25^oC) and pH 5.1 while varying substrate concentrations. For determination of K_m and V_{max}, assay mentioned in section 2.3.2.1.2 was applied.

2.3.2.1.4 Temperature Stability of Immobilized Glucose Oxidase

Effect of temperature on the enzyme activity of glucose oxidase was studied by changing temperature in range of 10-80 ^oC at constant glucose concentration. Activity assay was applied as described in section 2.3.2.1.2.

2.3.2.1.5 pH Stability of Immobilized Glucose Oxidase

Influence of pH on the enzyme activity of glucose oxidase was studied in the pH range between 4 and 11 at constant glucose concentration and 25^oC. The rest of the procedure was the same as mentioned in section 2.3.2.1.2.

2.3.2.1.6 Operational and Long-term Stability of Immobilized Glucose Oxidase.

To study on the repeated use of immobilized GOD, 40 activity measurements were performed on the same day at constant glucose concentration and 25^oC.

To evaluate long-term stability of immobilized GOD, activity of the electrode was monitored for 40 days at constant glucose concentration and 25^oC.

2.3.2.1.7 Protein Determination

The protein determination measurements were performed according to Lowry method [68]. The Lowry reagent was prepared as follows: 100ml (2% w/v) Na_2CO_3 in 0.1M NaOH, 1ml (2% w/v) sodium potassium tartarate in H_2O and 1ml (1% w/v) $CuSO_4$ in H_2O were mixed to prepare the Lowry reagent.

For the calibration curve, Bovine serum albumin (BSA) was used. 0.1mg/ml BSA in 0.85% NaCl standard solution was prepared. Different concentrations of BSA were prepared as 1ml. Then, 5ml Lowry reagent were added to BSA solutions and waited for 10 min for preincubation. 0.5ml (50%) folin-phenol reagent was added and waited for 30 min. After 30 min, the absorbance was measured at 750 nm for each solution.

2.3.2.1.8 Determination of Glucose in Juices

The immobilized glucose oxidase electrodes were used for determination of glucose amount in two kinds of Turkish orange juices. Orange juices were diluted with acetate buffer (pH 5.1) in 1:50 proportion. Activity assay described in section 2.3.2.1.2. was applied.

2.3.2.2 Polyphenol Oxidase (PPO) Immobilization

2.3.2.2.1 Immobilization Polyphenol Oxidase in Copolymer P(MBThi-co-Py)

For the immobilization of polyphenol oxidase (PPO), 4mg (0.4 mg/ml) PPO (1,530 units/mg), 10mg (1mg/ml) SDS, 0.072 M pyrrole, and 10 ml citrate buffer were mixed to prepare electrolysis solution. The rest of the procedure is the same as described glucose oxidase immobilization as mentioned in section 2.3.2.1.1. After electropolymerization, enzyme electrodes were washed in order

to remove unbound enzyme and SDS and kept in citrate buffer at 4°C when not in use.

2.3.2.2.2 Assay of Polyphenol Oxidase Activity

Polyphenol oxidase (PPO) activities for free and immobilized enzyme were determined according to Besthorn's Hydrozone Method [69]. In this method, 3-methyl-2-benzothiozolinone hydrazone (MBTH) reacts with the quinones produced to form red products instead of brown colored pigments in the absence of MBTH [70]. The reaction pathway is shown in figure 2.5 [71].

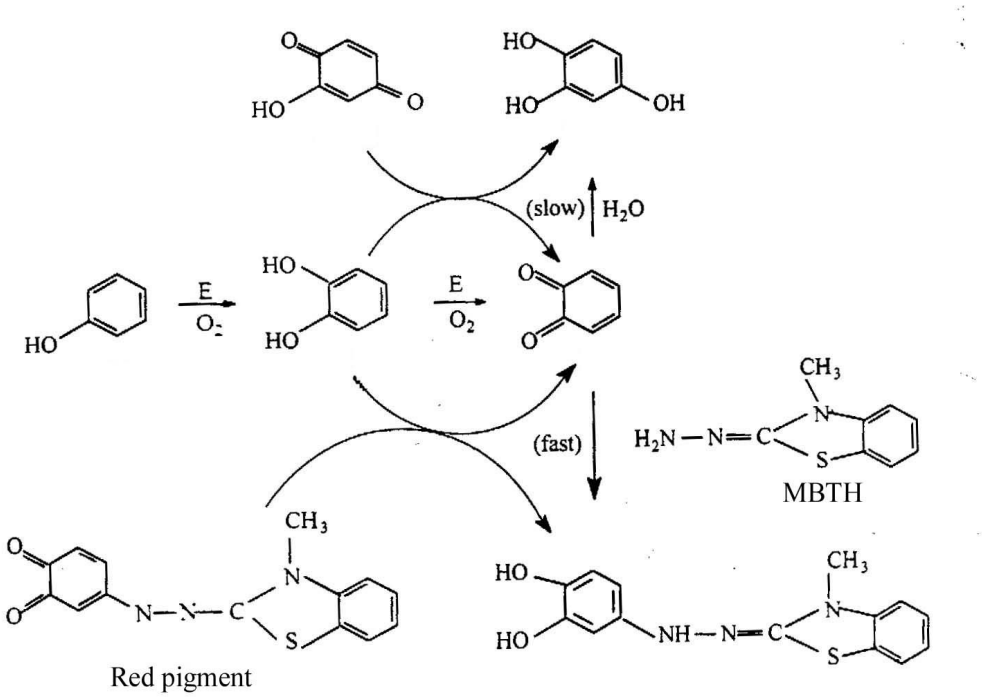


Figure 2.5 Reaction pathway for polyphenol oxidase

For the free polyphenol oxidase activity, 0.2 ml (0.015mg/ml) PPO was added to the mixture of 1.5 ml catechol in citrate buffer and 0.5 ml MBTH in ethanol. After addition of PPO, specific times (1, 2 and 3 min) were given so that reaction occurs. Then, 0.8 ml (5%) H_2SO_4 and 1 ml acetone were added. After shaking the mixture, absorbance was measured at 495nm.

For immobilized enzyme, 3ml catechol and 1ml MBTH were mixed and enzyme electrode was immersed for 5, 10 and 15 min at 25^{o}C. After removing the electrode, 1ml (5%) H_2SO_4 and 1ml acetone were added. Absorbance was measured at 495 nm. Quinone standart curve was used to define the enzyme activity. One unit of enzyme activity is expressed as the amount enzyme required to produce 1μmol of quinone per minute at pH 6.5 and 25^{o}C.

2.3.2.2.3 Kinetic Parameters for Immobilized Polyphenol Oxidase

Kinetic parameters (K_m, V_{max}) for free and immobilized PPO were investigated at constant temperature (25^{o}C) and pH (6.5) while varying substrate concentration. For the determination of K_m and V_{max}, assay mentioned in 2.3.2.2.2 was applied.

2.3.2.2.4 Temperature Stability of Immobilized Polyphenol Oxidase

Effect of temperature on the enzyme activity of polyphenol oxidase was studied by changing temperature in range between 10 and 80 ^{o}C at constant catechol concentration. Activity assay was applied as described in section 2.3.2.2.2.

2.3.2.2.5 pH Stability of Immobilized Polyphenol Oxidase

Influence of pH on the enzyme activity of polyphenol oxidase was studied in pH range between 2 and 11 at constant catechol concentration and 25^{o}C. The rest of the procedure was the same as mentioned in section 2.3.2.2.2.

2.3.2.2.6 Operational and Long-term Stability of Immobilized Polyphenol Oxidase.

To study the repeated use of immobilized PPO, 40 activity measurements were performed on the same day at constant catechol concentration and 25^{o}C.

To evaluate long-term stability of immobilized PPO, activity measurements were monitored for 40 days at constant catechol concentration and 25^{o}C

2.3.2.2.7 Protein Determination

Protein determination measurements were done according to Lowry method. The same procedure described in section 2.3.2.1.7 was used.

2.3.2.2.8 Determination of Phenolic Compounds in Red Wine

Immobilized polyphenol oxidase electrodes were used to analyze the concentration of phenolic compounds in two kinds of Turkish red wines, Brand K and Brand D. Total phenolic compounds in wines produced in Turkey were reported as 2000-3000 mg/L [72-74]. Red wines are diluted in a proportion of 1:3 with citrate buffer of pH 6.5 and activity assay was applied as described in section 2.3.2.2.2.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of Copolymer of MBThi with Pyrrole P(MBThi-co-Py)

3.1.1 Synthesis of Copolymer of MBThi with Pyrrole

Electrochemical polymerization of random copolymer of MBThi with pyrrole was achieved in the presence of SDS by constant potential electrolysis (Figure 3.1).

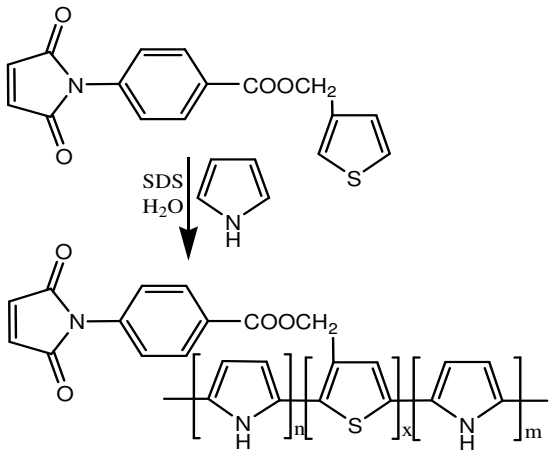


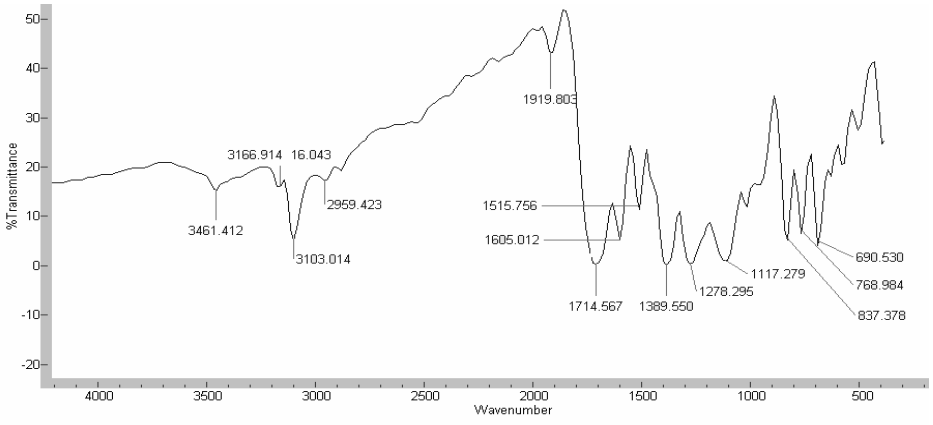
Figure 3.1 Electrochemical synthesis route for copolymerization.

3.1.2. Characterization of Copolymer of MBThi with Pyrrole

3.1.2.1 FT-IR Study

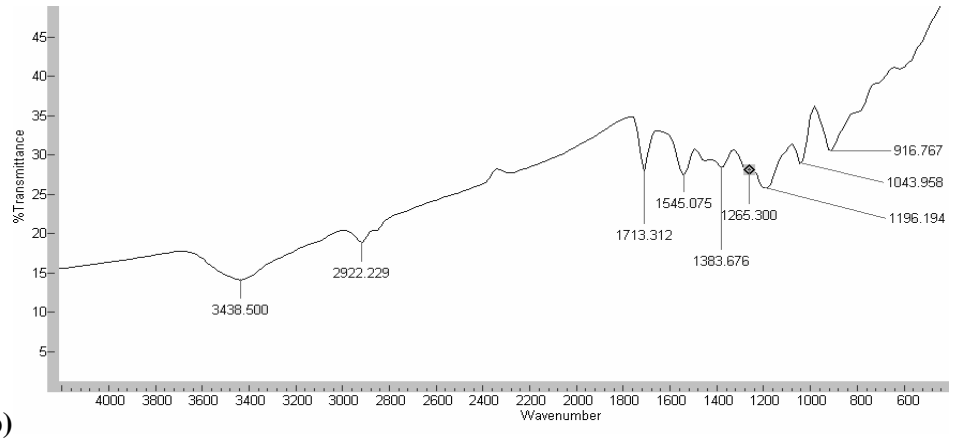
FT-IR spectrum of MBThi and P(MBThi-co-Py) are given in figure 3.2. FT-IR spectrum of monomer, MBThi, exhibits characteristic carbonyl peak at 1714 cm^{-1}. FT-IR spectrum of MBThi exhibits the peaks α-CH out of plane bending and α-CH stretching of thiophene ring at 768 and 3103 cm^{-1} respectively. Other peaks which belongs to MBThi are 2959, 1369, 1278, 1117, 837 and 690 cm^{-1}.

Copolymer has a peak around 1714 cm^{-1}. The carbonyl peak reveals that copolymerization occurred between MBThi and pyrrole since FTIR was run after washing of film with the solvent of MBThi.



(a)

Figure 3.2 FT-IR spectrum of (a) MBThi and (b) P(MBThi-co-Py)



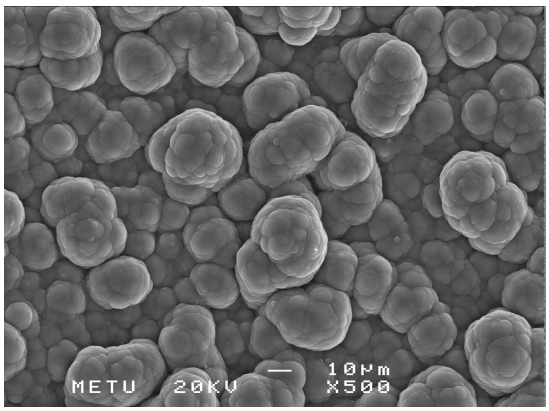
(b)

Figure 3.2 (Cont.)

Morphologies of Films

Scanning Electron Microscopy (SEM) was performed in order to examine the morphologies of polymer films since morphologies of films give information whether copolymerization was achieved or not. When examining the morphologies of films, it is observed that P(MBThi-co-Py) exhibited different morphology compared to polypyrrole. Polypyrrole has cauliflower like structure. During the copolymerization, this structure was altered. The SEM micrographs of PPy and P(MBThi-co-Py) were shown in figure 3.3.

(a)



(b)

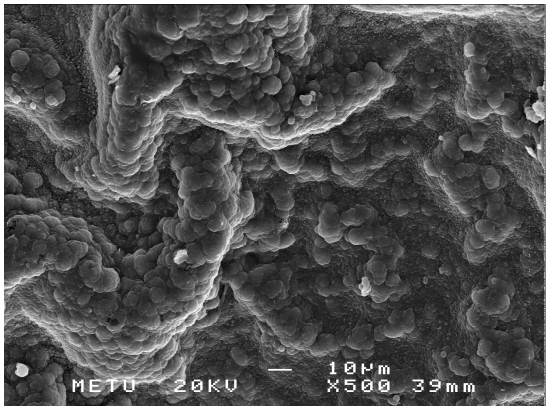


Figure 3.3 SEM micrographs of (a) PPy and (b) P(MBThi-co-Py)

3.1.2.3 Conductivities of the Films

Conductivity measurement is yet another characterization method. For that matter, conductivities of PPy and P(MBThi-co-Py) were compared. Standard four-probe technique was used for measuring the conductivities of polymers. Conductivities of PPy and P(MBThi-co-Py) were calculated as 7.4×10^{-3} and 7.3×10^{-6} S/cm respectively. Conductivity of PPy is 1000 times greater than that of P(MBThi-co-Py). Hence, copolymerization of MBThi with pyrrole is said to be achieved.

3.2 Immobilization of Enzymes

3.2.1 Glucose Oxidase (GOD) Immobilization

3.2.1.1 Kinetic Parameters of Immobilized GOD

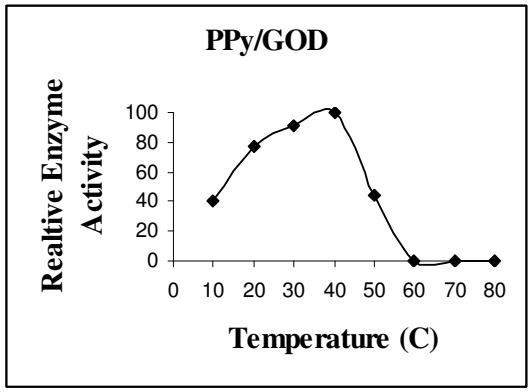
The effect of immobilization on the kinetic parameters was performed by measuring the activities of free and immobilized glucose oxidase at various concentrations of glucose, at constant temperature (25^{o}C) and pH (5.1). The values of kinetic parameters, V_{max} (maximum reaction rate) and K_m (affinity of enzyme to substrate), are shown in table 3.1**.** These parameters were calculated by the help of Lineweaver-Burk plot [75]. Kinetic behavior of glucose oxidase was changed by immobilization as expected. V_{max} values of immobilized GOD in both PPy and P(MBThi-co-Py) matrices were almost the same. However, K_m value of GOD in P(MBThi-co-Py) was 4.57 mM which was lower than that of GOD in PPy matrix. As a result, GOD in P(MBThi-co-Py) matrix has higher affinity to substrate compared to PPy matrix.

Table 3.1 Kinetic parameters of GOD.

	V_{max}		K_m (mM)
Free GOD	0.68	(μmol/min.mL)	3.04
PPy / GOD	0.040	(μmol/min.electrode)	11.8
P(MBThi-co-Py) / GOD	0.033	(μmol/min.electrode)	4.57

3.2.1.2 Effect of Temperature on GOD Activity

Working temperature affects the relative activity of enzyme electrodes. To examine the influence of temperature, the relative activity of enzyme electrodes was investigated at temperatures between 10-80^{o}C. The maximum activity was found to be 30^{o}C for free enzyme reported in a previous study [76]. The temperature of maximum activity of GOD in copolymer and PPy matrices was observed at 30 and 40 ^{o}C respectively (figure 3.4). On the other hand, the relative activity of immobilized enzyme decreases slowly after 30 ^{o}C for the copolymer matrix. However, the enzyme lost its activity completely at 60^{o}C in PPy matrix. As a result, copolymer matrix provides higher stability against high temperatures than the PPy matrix.



(a)

Figure 3.4 Effect of temperature on GOD activity immobilized in (a) PPy, (b) P(MBThi-co-Py) enzyme electrodes.

(b)

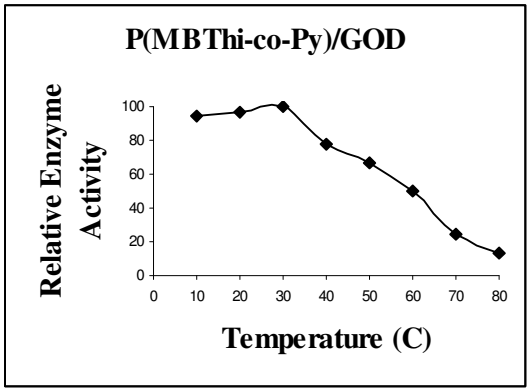


Figure 3.4 (Cont.)

3.2.1.3 Effect of pH on GOD Activity

Optimum pH of glucose oxidase in PPy and copolymer matrices was studied between pH 4 and 11. Free GOD had maximum activity at pH 5.5 reported in previous study [76]. The optimum pH of PPy and P(MBThi-co-Py) electrodes was shifted towards to the alkaline side compared with the free enzyme. This might be explained by the partitioning of protons [76]. As seen in figure 3.5, maximum activity was observed at same pH for both matrices.

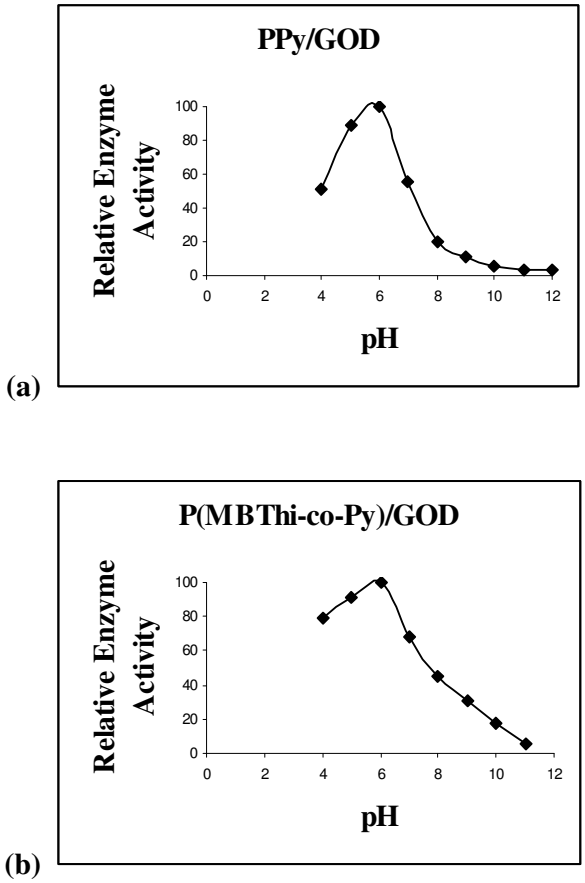
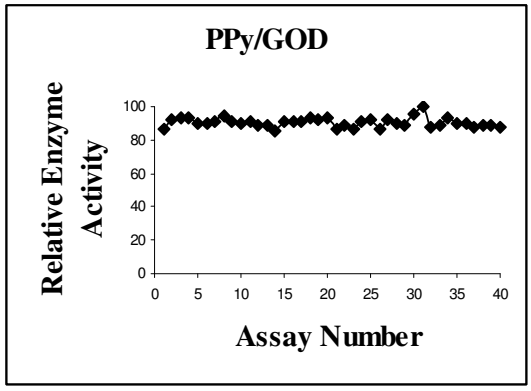


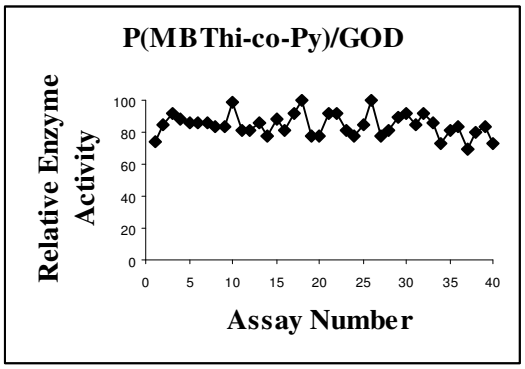
Figure 3.5 Effect of pH on GOD activity immobilized in (a) PPy, (b) P(MBThi-co-Py) enzyme electrodes.

3.2.1.4 Operational Stability of GOD Electrodes

To determine the operational stability of immobilized glucose oxidase, activity assay was performed in a day for 40 measurements. Activity of GOD in PPy matrix remained almost constant for 40 measurements. On the other hand, activity of GOD in copolymer matrix showed average 85% activity with some fluctuations for 40 measurements (Figure 3.6). Thus, PPy matrix provided better operational stability compared to copolymer matrix.



(a)

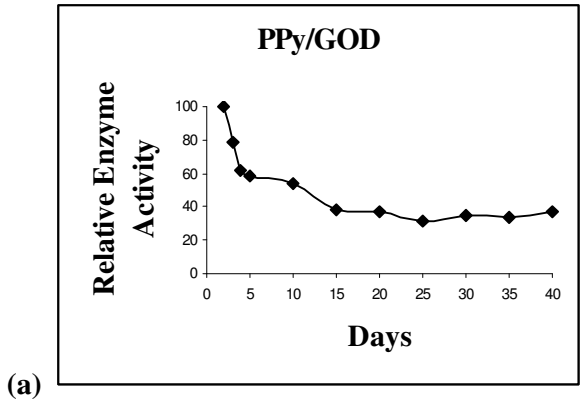


(b)

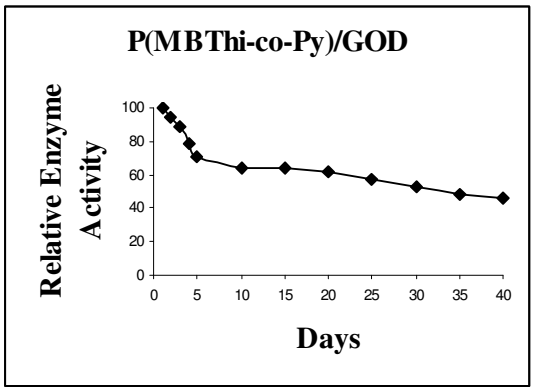
Figure 3.6 Operational stability of GOD immobilized in (a) PPy, (b) P(MBThi-co-Py) enzyme electrodes

3.2.1.5 Long-term Stability of GOD Electrodes

To evaluate long term stability of enzyme electrodes, their responses were monitored for 40 days. The responses of GOD enzyme immobilized in PPy and P(MBThi-co-Py) are given in figure 3.7. For GOD immobilized in both matrices, similar stability behaviors were observed end of 40 days. However, the stability provided by copolymer matrix was higher compare to PPy matrix.



(a)



(b)

Figure 3.7 Long-term stability of GOD immobilized in (a) PPy, (b) P(MBThi-co-Py) enzyme electrodes.

3.2.1.6 Protein determination

To determine the amount of immobilized protein, Lowry method was used. The calibration curve was prepared by using bovine serum albumin (Figure 3.8).

The results showed that 9.5×10^{-5} and 7.8×10^{-5} mg protein were entrapped in PPy and copolymer electrodes respectively.

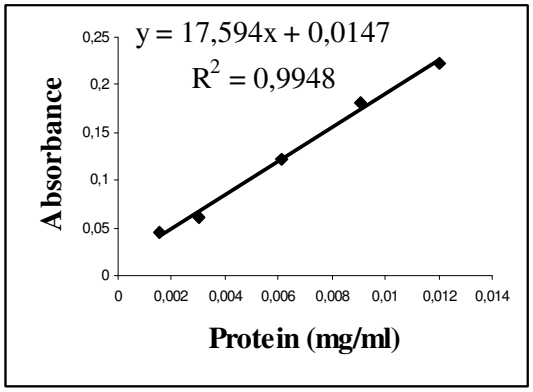


Figure 3.8 Calibration curve for protein determination.

3.2.1.7 Glucose in Orange Juices

The enzyme electrode was used for determining glucose concentration in orange juices. Two Turkish orange juices, brand D and brand M, were analyzed to investigate their glucose content by using P(MBThi-co-Py) enzyme electrode. Lane-Eynon analysis for glucose in the same juices was performed. In Table 3.2, comparable results were obtained for both methods. Brand M contains higher glucose content than Brand D.

Table 3.2 Glucose amount in two kinds of orange juices.

	Brand D (g/100ml)	**Brand M (g/100ml)**
Lane-Eynon Method*	1.50	2.15
PPy*	1.45	1.44
P(MBThi-co-Py)	1.56	2.04

*[76]

3.2.2 Polyphenol Oxidase (PPO) Immobilization

3.2.2.1 Kinetic parameters of Immobilized PPO

Kinetic parameters, K_m and V_{max} of free and immobilized polyphenol oxidase were investigated at constant temperature (25^oC) and pH (6.5) while varying substrate (catechol) concentration. Lineweaver-Burk plot was used to calculate K_m and V_{max} for both free and immobilized PPO. Kinetic parameters were given in table 3.3. It was observed that kinetic parameters of PPO were altered by immobilization as expected. K_m value of PPO in PPy matrix is lower than the one in P(MBThi-co-Py) matrix. Thus, the interaction between PPO and catechol in PPy matrix is stronger than the one for copolymer matrix since the lower K_m value means that the affinity between enzyme and substrate is higher.

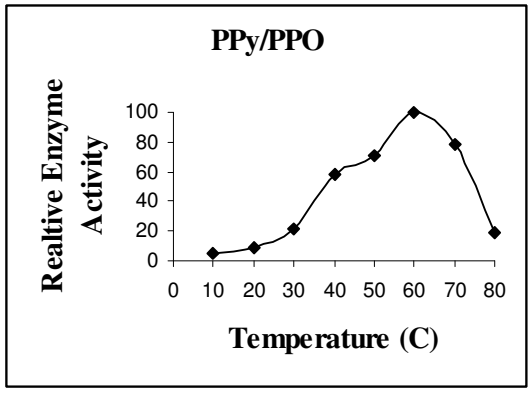
V_{max} value of PPO in P(MBThi-co-Py) is higher than that of PPO in PPy matrix. Lower V_{max} of PPO in PPy matrix can be due to the lower amount of enzyme entrapped in the matrix.

Table 3.3 Kinetic parameters of PPO.

	V_{max}		**K_m (mM)**
Free PPO	0.1	(μmol/min.mL)	0.14
PPy/PPO	0.0064	(μmol/min.electrode)	17
P(MBThi-co-Py)/PPO	0.0088	(μmol/min.electrode)	40

3.2.2.2 Effect of Temperature on PPO Activity

The activity of immobilized PPO obtained in a temperature range of 10-80^{o}C was expressed as relative activity. The maximum activity was found to be 40^{o}C for free enzyme reported in a previous study and lost its activity completely at 50^{o}C [58]. For immobilized polyphenol oxidase, the activity of enzyme increases with increasing temperature (Figure 3.9). The maximum activity of enzyme in PPy and P(MBThi-co-Py) was observed at 60 and 70 ^{o}C respectively. After 70 ^{o}C, the response of PPO in copolymer matrix decreases suddenly to zero. It is possible that enzyme in the copolymer matrix denatures completely at 80 ^{o}C.



(a)

Figure 3.9 Effect of temperature on PPO activity immobilized enzyme in (a) PPy, (b) P(MBThi-co-Py) enzyme electrodes.

(b)

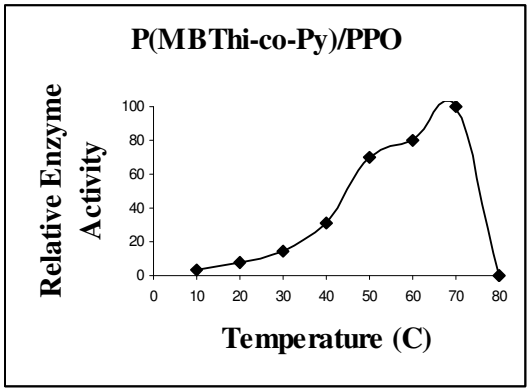
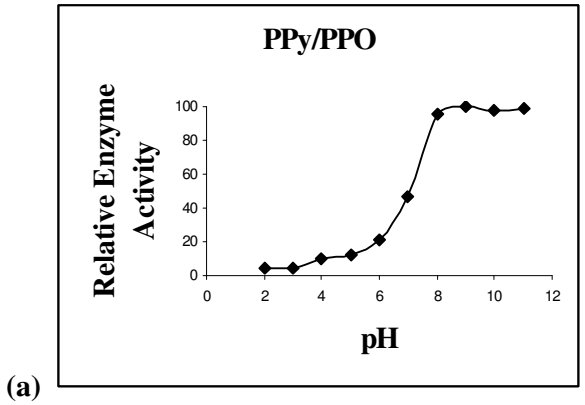


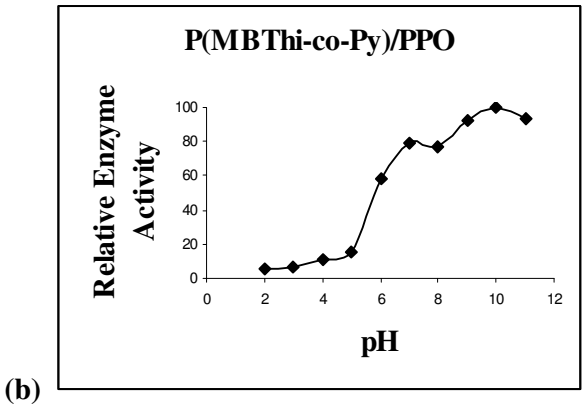
Figure 3.9 (Cont.)

3.2.2.3 Effect of pH on PPO Activity

The effect of pH on the activity of immobilized PPO was investigated between pH 2 to 11 and results were given in figure 3.10. Free PPO had maximum activity at pH 5 which was reported in a previous study [58]. The pH of maximum activity for PPy and P(MBThi-co-Py) electrodes were shifted towards to the alkaline side in compare to the ones for free enzyme. This might be explained by the partitioning of protons [56]. These electrodes exhibit high stability towards higher pH. For PPy electrode, there is no change in the enzyme activity between pH 8 and 11. P(MBThi-co-Py) electrode also indicates almost the same behavior. This behavior shows that these electrodes can protect PPO against high hydroxyl concentrations.



(a)



(b)

Figure 3.10 Effect of pH on PPO activity immobilized in (a) PPy, (b) P(MBThi-co-Py) enzyme electrodes.

3.2.2.4 Operational Stability of PPO Electrodes

Operational stability is an important parameter for immobilized enzymes. Operational stability of enzyme electrodes in terms of repetitive use was studied by doing 40 measurements in the same day. The results are given in figure 3.11. For the copolymer matrix, activity remained constant almost for 40 measurements. PPy enzyme electrode lost its activity continuously during

measurements and at the end, it lost 50% of its activity. As a result copolymer matrix provides higher operational stability than PPy matrix.

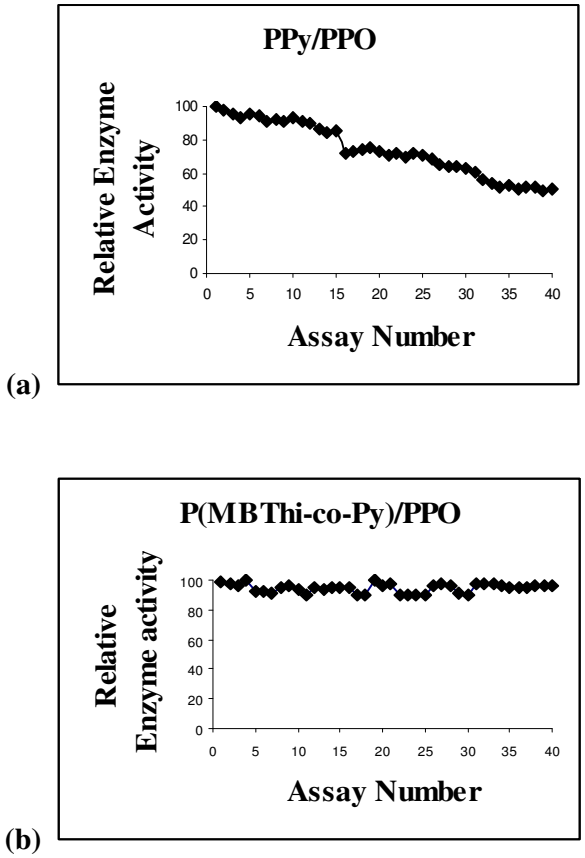


Figure 3.11 Effect of repetitive use on PPO activity immobilized in (a) PPy, (b) P(MBThi-co-Py) enzyme electrodes.

3.2.2.5 Long-term Stability of PPO Electrodes

Long-term stability of PPO entrapped in PPy and copolymer matrices was determined for 40 days. The responses of PPO enzyme immobilized in PPy and P(MBThi-co-Py) are given in figure 3.12. PPy enzyme electrode

lost 45% of its activity in the first 5 days. No further activity loss was observed within the next 35 days. Copolymer enzyme electrode lost about 50% of its activity in 25 days. There was no activity loss therein.

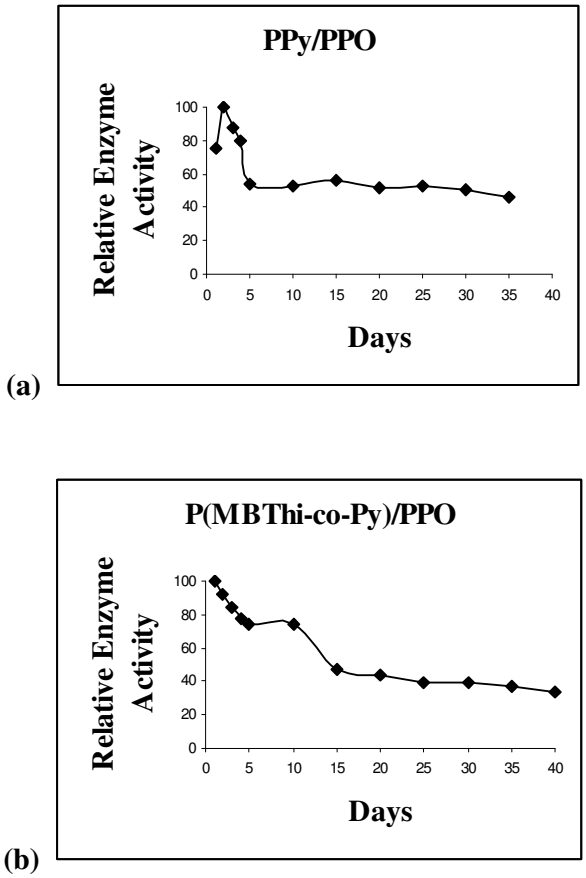


Figure 3.12 Long-term stability of PPO immobilized in (a) PPy, (b) P(MBThi-co-Py) enzyme electrodes.

3.2.2.6 Protein Determination

To determine the amount of immobilized protein, Lowry method was used. The calibration curve was prepared by using Bovine Serum Albumin (Figure 3.13).

Results showed that $2.4x10^{-4}$ and $3.3x10^{-4}$ mg protein were entrapped in PPy and copolymer electrodes respectively.

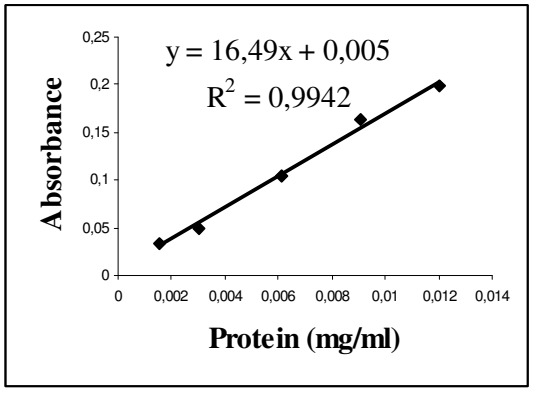


Figure 3.13 Calibration curve for protein determination.

3.2.2.7 Phenolic Compounds in Red Wine

Immobilized polyphenol oxidase electrodes were used to analyze the concentration of phenolic compounds in two kinds of Turkish red wines, Brand K and Brand D.

Results are given in table 3.4. Concentration found for phenolic compounds using free PPO is much lower compared to immobilized enzyme electrodes since wines include inhibitors of PPO. In literature, benzoates act as inhibitors for free PPO therefore, they inhibited PPO

before it completes enzymatic reaction. On the other hand, enzyme electrodes protect PPO against inhibitors. The phenolic amount was found as 4833 mg/L for Brand K and as 1083 mg/L for Brand D by P(MBThi-co-Py) enzyme electrode.

Brand K contains more phenolics compared to Brand D. High amount of phenolics in Brand K is responsible for the bitter taste of the wine.

Table 3.4 Phenolic compounds in Turkish red wines.

	Brand K	**Brand D**
Free PPO*	220mg/L	270mg/L
PPy/PPO*	4000mg/L	2200mg/L
P(MBThi-co-Py)/ PPO	4833mg/L	1083mg/L

*[60]

CHAPTER IV

CONCLUSIONS

Syntheses of a random copolymer of MBThi with pyrrole were achieved via constant potential electropolymerization. Glucose oxidase and polyphenol oxidase were succesfully immobilized in PPy and P(MBThi-co-Py) matrices.

Although immobilized GOD for both matrices has almost the same Vmax value, P(MBThi-co-Py) has higher affinity to substrate. Immobilized GOD reveals maximum activity for P(MBThi-co-Py) and PPy matrices at 30 and 40^{o}C respectively. Immobilized GOD reveals maximum activity for PPy and P(MBThi-co-Py) matrices at pH 6. PPy matrix showed good operational stability compared to P(MBThi-co-Py) matrix however, the long-term stability of P(MBThi-co-Py) matrix was higher than that of PPy matrix. GOD electrodes were utilized successfully for the determination of glucose in orange juices.

Immobilized PPO for both matrices has comparable V_{max} value. Unlike P(MBThi-co-Py), PPy matrix has lower K_m value. Therefore, the affinity between enzyme and substrate is higher in PPy matrix. Immobilized PPO reveals maximum activity for PPy and P (MBThi-co-Py) matrices at 60 and 70^{o}C respectively. Both matrices protect PPO against high OH concentration. P(MBThi-co-Py) matrix showed better operational stability than

PPy matrix. PPO electrodes were used successfully to determine the concentration of phenolic compounds in red wines

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