ANALYTICAL APPLICATIONS OF IMMOBILIZED TYROSINASE FOR PHENOL DETERMINATION

By

KURUVILLA ZACHARIAH

Bachelor of Science
University of Kerala
Trivandrum, India
1966

Master of Science
Ravishankar University
Raipur, India
1970

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Thesis approved:

Horacio A Mottola
Thesis Adviser

Warren T Ford

[signature]

Ziad El Rassi

Satyanarayan Nandi

Norman N. Durham
Dean of the Graduate College

PREFACE

With a view to impart clarity and comprehension to the reader, this thesis is divided into six chapters. Chapter I introduces the subject of the study with special reference to immobilized enzymes and continuous-flow sample processing systems. The methods already reported in the literature regarding the immobilization of the enzyme tyrosinase are briefly reviewed in Chapter II. Chapter III is a critical review of the analytical chemistry of phenols for water samples. Chapter IV and V describe the method of immobilization of tyrosinase, the construction of open tubular enzyme reactors, and the continuous-flow determination of phenols. The conclusions drawn from this study are presented in Chapter VI.

I like to express my deep and sincere gratitude and appreciation to my thesis adviser, Dr. Horacio A. Mottola. He not only guided me through courses and research, but also introduced me and my son to the wonderful experience of fishing. Dr. Mottola's dedication to teaching and research and commitment to excellence will always be a model for me. I extend my thanks to the members of my advisory committe, Dr. W. T. Ford, Dr. N. Purdie, Dr. Z. El Rassi, Dr. S. Nandi and to Dr. P.W. Geno for their time and advice.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AA-CPG	Arylamine controlled-pore glass
CPG	Controlled-pore glass
DOPA	Dihydroxy phenyl alanine
FIA	Flow injection analysis
LOD	Limit of detection
MDQ	Minimum detectable quantity
OTR	Open tubular reactor
PVP	Poly(4-vinylpyrididne)
rsd	Relative standard deviation
SBSR	Single bead string reactor

CHAPTER I

INTRODUCTION

The quality of the air we breathe and the water we drink has become a global concern. Regulatory agencies such as the United States Environmental Protection Agency (EPA) have established stricter limits on the amount of pollutants acceptable in atmospheric air and drinking water. Phenolic materials are found in a wide range of effluents that can contaminate water supplies and the United States EPA's list of priority pollutants contains eleven phenolic compounds [1,2]. The agency has also set a limit on phenol at one $\mu g\ l^{-1}$ (ppb) for drinking water [3]. There is growing demand on environmental laboratories to analyze more and more water samples to assure the quality of water for public use.

Considerable saving in time and manpower can be achieved if routine analytical procedures can be automated. One of the means by which this can be realized is by implementing continuous-flow systems. A large number of samples can be analyzed within a short period of time by the continuous-flow sample processing technique. Enzymes have long been recognized as catalysts that can react under mild conditions in neutral aqueous solutions at normal

temperature and pressure [4]. An enzyme that can selectively catalyze the oxidation of certain mono and dihydric phenols is tyrosinase.

When this study was undertaken, the intention of the author was to develop a method for the determination of phenols in water samples by making use of the selectivity of tyrosinase towards phenols. Another idea was to employ a continuous-flow sample processing system to meet the additional demand on the laboratories to analyze large numbers of water samples.

Enzyme Catalysts

Enzymes are biochemical catalysts synthesized *in vivo* by living organisms. As catalysts, enzymes affect the rate at which the composition of the system attains equilibrium in a reaction. Enzymes are high molecular weight proteins. Each enzyme is characterized by a specific geometric area called the active site into which only one or a very limited number of substrates will fit [5]. Thus enzymes are highly selective or often specific towards substrates which prevents interferences from other species present in the reaction. Enzyme catalyzed reactions can be conveniently used to determine enzyme activities and substrate concentrations.

Purified enzyme preparations are not only expensive but also scarce. Large quantities of enzymes are often

difficult to obtain. Unlike most heterogeneous catalysts, enzymes are soluble and their aqueous solutions lose the catalytic ability rapidly. Since they cannot be recovered from solutions, enzymes cannot be reused. These limitations have precluded the use of enzymes as analytical reagents in many chemical and biochemical reactions.

Immobilization of Enzymes

Developments in immobilization technology have led to a big surge in the analytical applications of enzymes to a wide variety of reactions. Immobilization is the confinement or localization of the enzyme in such a manner that it remains physically separated from the substrate and the products of the enzyme-catalyzed reaction [6]. Immobilized enzymes have become very popular as biochemical catalysts because of a number of advantages such as retention of activity with time, insensitivity to inhibitors and activators, broad pH range, and particularly reusability. Since immobilized preparations can be used over and over again without substantial loss of activity, their use is also cost effective.

Continuous-flow Sample Processing

Continuous-flow sample processing is a rapidly growing technique that has found a wide variety of applications in many fields of analysis including clinical, agricultural,

food and beverages, and environmental [7]. It is a versatile technique because it can handle gaseous as well as liquid systems, and can easily incorporate on-line ancillary steps [8]. A very convenient and simple to use form of this solution manipulation technique is Flow Injection Analysis popularly abbreviated as FIA.

Flow Injection Analysis

FIA is an unsegmented continuous-flow sample processing system which utilizes pumps or gravity to move the carrier stream, reagents, and samples through a manifold composed of narrow tubes connecting various components such as sample injection valve, extractor, reactor and detector. The sample is intercalated into the carrier stream and forms a "plug" between segments of the moving stream. Although FIA cannot be precisely defined, the important features on which it is based are: sample injection into an unsegmented carrier stream, controlled dispersion, and reproducible timing of the movement of the concentration gradient through the flow channel and the detector [9]. Repeated injection of fixed sample volumes into an exactly specified flow system will yield reproducible responses as measured by the detector [10]. The signals from the detector can be recorded on suitable recording devices in the form of flow injection peaks. The peak height is directly proportional to the analyte concentration; although sometimes it may be

necessary to integrate the area under the peak. FIA manifolds can be designed to meet individual needs using low cost components and can also be miniaturized. These attractive features have helped FIA to develop into a powerful analytical tool capable of processing a large number of samples with acceptable precision and accuracy [11].

Enzyme Reactors

Immobilized enzymes can be conveniently incorporated into continuous-flow systems by means of "enzyme reactors". Although several types of reactor configurations such as packed columns, stirred tank and single bead string reactors have been used, open tubular reactors (OTR), with the enzyme immobilized on the inner walls, first introduced by Hornby and co-workers [12,13] are among the most widely used reactor type today [14]. Low pressure drop, permitting lower flow rate, high throughput of number of samples and low dispersion when coiled are among the advantages of these reactors. The main draw back of the original reactors fabricated from nylon tubing by Hornby et al. was low catalytic surface area. Horvath et al. [15,16] have tried to increase the surface area of such reactors by providing an annulus of silica or polymer. Iob and Mottola [17], and Kojima et al. [18] have been able to increase the surface area of glass capillary open tubular reactors by growing

"whiskers".

A simple and quicker method of preparing OTRs has been developed by Gosnell et al. [19] by thermally embedding controlled-pore glass on the inner walls of Teflon and Tygon tubing. Their procedure has not only enhanced the surface area but also provided more uniform coverage of the enzyme when immobilized.

Though enzymes have been extensively used in clinical chemistry, their use as a powerful analytical tool, especially in environmental analysis is not so wide spread. The enzyme tyrosinase has been successfully immobilized by other researchers. But no serious effort has been made to use it for the determination of phenols in water supplies. This study is an attempt to use immobilized tyrosinase reactors in continuous-flow systems using spectrophotometric and amperometric detection methods to determine trace amounts of phenols in water.

CHAPTER II

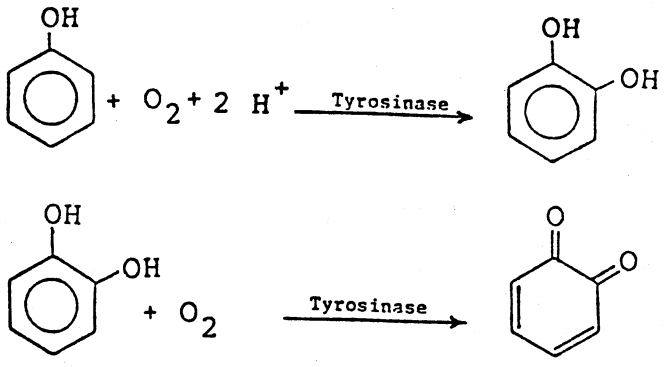
SOURCES, PROPERTIES, AND METHODS OF IMMOBILIZATION OF TYROSINASE

Sources and Properties

The enzyme tyrosinase (E.C. 1.10.3.1, monophenol, monooxygenase, CA Registry No. 9002-10-2) has been purified from various sources such as mushrooms [20-24], potato tubers [25], Neurospora Crassa [26], mammalian melanoma tumors [27], and frog epidermis [28-29]. Most of the work reported in the literature has been done with tyrosinase from edible mushroom (Agaricus bispora). This enzyme is presumed to exist as a tetramer of approximate molecular weight 126,000 [24]. It contains four atoms of copper per molecule which corresponds to a copper content of 0.21% [23].

Tyrosinase is a bifunctional enzyme having both cresolase and catecholase activities [30]. In the presence of molecular oxygen, it catalyzes the hydroxylation of phenol, cresol and other monohydroxy derivatives of benzene to o-dihydroxy compounds (cresolase activity) and subsequently the dehydrogenation of these compounds to the corresponding o-quinones (catecholase activity).

The oxidation of ordinary phenol catalyzed by tyrosinase is shown below which illustrates the activities of the enzyme.



Methods of Immobilization

One of the early attempts to immobilize tyrosinase that has been reported in the literature was by Wykes et al. [31]. They were able to immobilize mushroom tyrosinase by coupling the enzyme to DEAE-Cellulose previously treated with 2,4-dichloro-6-amino-s-triazine. The immobilized enzyme was used to convert L-tyrosine to L-DOPA (3,4-dihydroxy phenyl alanine). About 75% of the enzyme activity has been reported to be lost after being used for 24 hours with 1.65×10^{-3} M tyrosine.

May and Li [32] immobilized tyrosinase by encapsulation in a liquid membrane emulsion consisting of a surfactant, a high molecular weight amine and a high viscocity hydrocarbon. The emulsion was then dispersed in an aqueous

solution of phenol when the substrate diffused through the hydrocarbon phase and was oxidized by the catalytic action of the enzyme. The oxidation products accumulated in the liquid membrane phase. Although very little leakage of the enzyme into the external layer was noticed, there was a decrease in the reactivity of the enzyme with time.

Another approach to the immobilization of tyrosinase was the entrapment of the enzyme on collagen membrane. Letts and Chase [33] successfully accomplished this and used the preparation in a plug flow reactor for the synthesis of L-DOPA after chemical modification with glutaraldehyde, ethyl acetimide or dimethyl adipimate. The chemical modification was employed to make the lysine ε-amino groups less reactive with the view of reducing the reaction inactivation of the enzyme. The authors were only partially successful in their attempt.

Schiller et al. [34] carried out the immobilization of tyrosinase in an acrylamide gel cast around a basket-woven platinum screen. In this electrochemical system, the immobilized enzyme was used to oxidize phenol to o-benzoquinone and then the product was reduced in the presence of hexacyanoferrate(II).

Diazo coupling was the method employed by Vilanova et al. [29] for the immobilization of the enzyme to inert supports. Aryl amine groups of Enzacryl-AA (a poly-acrylamide based support) and CPG-AA (a controlled pore glass support) were first diazotized and frog epidermis

tyrosinase was covalently coupled to these supports. The immobilized enzyme was used in stirred tank and packed bed column reactors for the production of L-DOPA. The immobilized preparation stored at -30 ^{o}C in the lyophilized form was found to be 100% active even after being stored for five years.

In a recent paper Soklovskii and Kovalenko [35] have reported the immobilization of enzymes by adsorption on alumina and carbonized alumina supports. 0.5-2% carbon content in the support was found to be optimal for tyrosinase. Less than 5% of the original activity was lost when the immobilized enzyme was stored for twelve days.

Immobilization of the enzyme by entrapment within liposome membrane is the method reported by Miranda et al. [36]. Various kinetic studies were conducted on the immobilized enzyme with a view to using it either as a reactor or as a tool for enzyme therapy. No data regarding the long term stability of the immobilized enzyme are available.

Hall et al. [37] have also immmobilized tyrosinase by physical entrapment of the enzyme dissolved in sodium phosphate buffer on Hybond-N-nylon membrane folded around a graphite foil. This enzyme electrode was operated in chloroform to determine p-cresol. Since the enzyme is insoluble in chloroform, it remained as a thin aqueous film on the hydrophilic support.

Entrapment on polyacrylamide gels and on various

membranes have been the most common methods of immobilization of enzyme tyrosinase. L-DOPA, an oxidation product of L-tyrosine by tyrosinase is a drug of choice in the treatment of Parkinson's disease. L-tyrosine is present in human blood serum and biochemists were interested in the synthesis of L-DOPA in situ by implanting tyrosinase immobilized membranes similar in properties to those present in the human body. Tyrosinase immobilized by covalent coupling on various inert supports have been found to be stable. Although most of the studies have been directed towards L-tyrosine as a substrate, a few have used phenols as substrates. Enzyme electrodes prepared by the entrapment of tyrosinase have been used by Schiller et al. [34] to determine phenols and by Hall et al. [37] to determine p-cresol.

CHAPTER III

ANALYTICAL CHEMISTRY OF PHENOLIC SPECIES FOR WATER SAMPLES

Introduction

In addition to air, water is the most important material needed for sustaining life. Therefore, it is not only the amount of water available but also the quality of water, that is of great importance. Water dissolves various substances when it contacts and enters the soil. In addition to natural pollutants such as plant and animal matter, domestic, agricultural and industrial waste products also contaminate water supplies.

The general public has become increasingly aware of the problems related to environmental pollution especially that of water supplies. This has resulted in positive responses from the politicians. The establishment of the Environmental Protection Agency (EPA) of the United States and the introduction of Environmental Quality Directives as part of legislation in the European Economic Community can be considered outcomes of such responses.

Phenols are a class of organic compounds whose common functional group is the hydroxyl group. The hydroxyl group is attached to a benzene ring, which may be a single

isolated benzene ring or a part of a condensed ring structure. Other functional groups also may be present in a phenol. Most laboratory studies have concentrated on monohydric phenols such as phenol, cresols and xylenols. The toxicity of these compounds are similar [38] and in many studies, ordinary phenol is used as a model compound.

Phenols are considered pollutants because they increase the oxygen demand of water thereby depriving aquatic life of essential oxygen [39]. Chlorophenols are produced when phenol contaminated water is chlorinated in the purification process. These derivatives impart an objectionable taste to drinking water even at such low concentration as several parts per billion. The eleven phenolic compounds listed as priority pollutants of water by the U.S. EPA [1,2] are: (1) phenol, (2) p-nitrophenol, (3) 2,4-dinitrophenol, (4) o-chlorophenol, (5) o-nitrophenol, (6) 2,4-dimethylphenol, (7) 4-chloro-3-methylphenol, (8) 2,4-dichlorophenol, (9) 2-methyl-4,6-dinitrophenol, (10) 2,4,6-trichlorophenol, and (11) pentachlorophenol.

Phenolic compounds are found in a wide range of effluents but are commonly associated with coke production, synthetic resin manufacture, petroleum refining, chemical industries, textiles, tanning, iron and steel, glass manufacture, and rubber processing [40].

This review aims at a critical evaluation of the different methods reported in the literature for the determination of phenolic species, especially those listed

as priority pollutants of water. These have been categorized mainly into optical and chromatographic methods. An attempt has been made to include the most relevent techniques that have been reported in the literature during the past 20 years. However, this is not intended to be an exhaustive review.

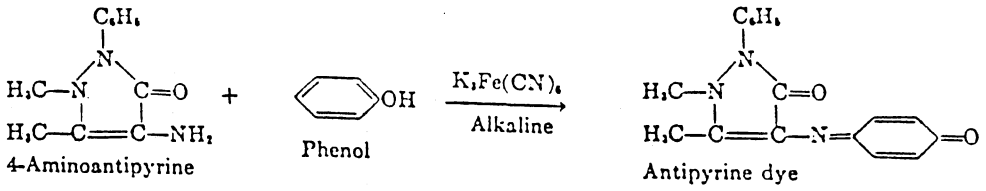
Standard Methods

Regulatory agencies and organizations have set forth guidelines for the determination of pollutants in water supplies. The "Standard Methods for the Examination of Water and Wastewater" [41] published by the American Public Health Association, the "Examination of Water for Pollution Control" [42] published on behalf of the World Health Organization, the American Society for Testing and Materials (ASTM) method D-1783-87 [43], and the U.S. EPA "Methods for Chemical Analysis of Water and Wastewater", Storet No. 32730 [44] describe almost identical methods for the determination of phenols. Three standard methods are described: two spectrophotometric methods and one gas chromatographic method. At least one distillation of the sample is suggested to purify phenols from non-volatile impurities.

Spectrophotometric methods

The colorimetric methods are based on the formation of antipyrine dyes by the reaction of 4-aminoantipyrine

(4-AAP), also known as 4-aminophenazone, with phenolic compounds in the presence of potassium hexacyanoferrate(III).



An ammonia-phosphate buffer has been recommended for use in the standard methods. The "Standard Methods for the Examination of Water and Wastewater" [41] suggests a pH of 7.9 ± 0.1, while the other three methods [42-44] recommend a pH of 10.0 ± 0.2. Goulden et al. [45] have studied the effect of pH and have reported an optimum value of 10.8 ± 0.5. They have used a buffer system consisting of K_2CO_3, boric acid and KOH. In a study of the factors that influence the condensation of 4-AAP with phenols, Faust and Mikulewiez [46,47] have stated that the color formation of the antipyrine dye is dependent upon the amount and type of base used. They have also noticed that an ammonia-phosphate buffer system was the most stable and gave higher molar absorptivity values at pH 8 when compared to pH 10. At pH 8, most of the phenols are partially converted to phenolate ions and it is easier to oxidize phenolate ions to quinones than the phenol itself. When the pH is increased to 10, there is increased competition between OH^- ions and the benzene ring for the $4\text{-}AAP^+$

which results in a reduction of the electrophilic character of the 4-aminiantipyrine.

Direct Method. The absorbance of the solution containing the dye is measured at 510 nm. The aqueous solution of the dye is not very stable and therefore the readings are to be taken within one hour of solution preparation.

Chloroform extraction method. The antipyrine dye is extracted from the aqueous solution with chloroform and its absorbance is measured at 460 nm. This method is more sensitive than the direct method and can be used to determine phenol concentrations in the $\mu g\ l^{-1}$ (ppb) range.

The spectrophotometric procedure measures a mixture of phenolic compounds which may have different rates of conversion to the antipyrine dye and different molar absorptivites. Therefore the response cannot be used to determine the "total phenol" concentration. It gives only the minimum concentration of phenols in terms of the equivalent concentration of ordinary phenol (C_6H_5OH) yielding the same response.

The 4-AAP method determines phenol, ortho and meta substituted phenols. If the pH conditions are properly maintained, those para substituted phenols in which the substituent group is carboxyl, methoxy, halogen or sulfonic

acid can also be determined. Phenols with alkyl, aryl, benzoyl, nitro, nitroso or aldehyde groups attached to the para position cannot be determined by this method.

Gas Chromatographic Method

This method involves the separation of phenolic compounds on certain gas chromatographic columns and their subsequent determination using a flame ionization detector. The peak area or the peak height of each component is measured and compared with that of a known standard. It is a direct aqueous injection procedure and can be applied to water samples containing more than one ug of phenol per ml.

A 3.2 mm by 3 m stainless steel column packed with 60/80 Chromasorb W coated with 20% by weight of Carbowax 20M-TPA is used. Isothermal elution at 210 + 0.2 ^{o}C is recommended. Elution of phenols occurs in the following order: (1) o-chlorophenol, (2) phenol and o-cresol, (3) m- and p-cresol, (4) 2,3-, 2,4-, 2,5-, and 2,6-dichlorophenols, (5) m- and p-chlorophenols and (6) 3,4-dichlorophenol.

This method though not as sensitive as the chloroform extraction method is capable of determining the para substituted phenols that cannot be determined by the 4-AAP method. Table I summarizes the three standard methods for the determination of phenols.

TABLE I

SUMMARY OF STANDARD METHODS FOR THE DETERMINATION OF PHENOLS

Method	Working concentration range	pH	Time required	Interferences	Advantages	Disadvantages
4-AAP Direct	1-500 $\mu g\ ml^{-1}$	7.9 or 10.0	0.25 to 1 hour	Turbidity, phenol decomposing bacteria oxidizing agents, reducing agents, oils and tars.	Speed of determination. Stable reagents. Applicable over wide concentration range.	All para substituted phenols cannot be determined. Values reported give only minimum concentration of phenol. 10-20% loss during distillation
4-AAP Chloroform extraction	2-200 $\mu g\ ml^{-1}$	"	1-2 hours	Same as above	All of the above. More sensitive than direct method.	All of the above. Extraction step needed before determination.
Gas Chromatography	1-800 $\mu g\ l^{-1}$	neutral or slightly acidic	-	Non-phenolics with same retention time as phenolics. Some chlorophenols form salts under alkaline conditions. "Ghost" interferences from previously analyzed samples.	Direct aqueous injections. Each component including para substituted phenols can be identified and determined.	Needs to inject water between samples to remove "ghost" interferences

Sensitivity, Limit of Detection, and Minimum Detectable Quantity

Two important figures of merit in analytical determinations are the sensitivity and the limit of detection. Sensitivity usually indicates the response of the instrument to changes in the analyte concentration and is expressed as the slope of the calibration curve, i.e., the change in analytical signal per unit change in analyte concentration. This is the definition of sensitivity accepted by the International Union of Pure and Applied Chemistry (IUPAC). The limit of detection (LOD) indicates the lowest analyte concentration that can be reported with a specified degree of confidence. It is defined as the analyte concentration that gives an analytical signal equal in magnitude to a confidence factor, k (usually k = 3) times the standard deviation of the blank signals. It has to be expressed in concentration units derived from the calibration curve. Alternatively, it can be expressed as the analyte concentration when the signal to noise ratio (S/N) = k. A value of k = 3 implies risk of 7% false positive and false negatives results [48]. A few authors have used the formula $2\ t\sqrt{2}(s_w)$ where, t is the Student's single sided t at a confidence level of 90%, and s_w the standard deviation of the blank, to calculate the limit of detection.

One problem that was encountered while reviewing the

literature for phenol determinations was that the terms sensitivity and limit of detection were used indiscriminately; often using the term sensitivity in place of limit of detection. Some of the papers in the gas and liquid chromatographic methods, have reported amounts in ng and pg and termed them as detection limits. One paper defines such a quantity as the amount which yields a signal to noise ratio of 2. Whenever such detection limits are encountered in this review, they are reported as the minimum detectable quantity (MDQ) and expressed in mass units. Many papers have reported the limit of detection without mentioning how it has been determined. When the method of determination has been mentioned, it was included in parentheses while quoting the limit of detection of a method. Otherwise, just the number given is quoted.

Optical Methods

The most common and widely used spectrophotometric technique for the determination of phenols is the 4-AAP method described as one of the standard methods. Two other spectrophotometric methods frequently employed are the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method and the ultraviolet-ratio (uv-ratio) method. The absorbance due to the colored product of phenols with another reagent is measured in most of the other methods described in the literature. Higher order derivative spectroscopy, Raman

spectroscopy and spectrofluorimetric techniques have also been used in the determination of phenols.

4- Aminoantipyrine method

The chemistry and other details of the 4-AAP method have been described earlier. A brief review of some of the papers describing modifications and improvements to the method is presented here. A summary of the observations is given in Table II.

An automated system which incorporates distillation and solvent extraction has been described by Goulden et al. [45]. Concentration of the antipyrine dye by using two chloroform extractions and optimization of conditions have improved the limit of detection of the method to 0.2 µg l^{-1} based on a signal to noise ratio of 2.

Afghan et al. [49] have found that although petroleum ether, benzene and chloroform could quantitatively extract highly substituted phenols, these solvents could not quantitatively extract phenol and cresols. They have reported that n-butyl acetate and isoamyl acetate are better solvents for extraction of phenol and cresols from water samples. Using an automated system with an on-line extraction step, they have reported 0.5 µg l^{-1} as the lower limit of detection. However, no mention has been made about the method by which this value has been determined.

Norvitz and Keliher [50] have studied the interferences

of aromatic amines and formaldehyde with the 4-AAP method for phenol. Aromatic amines produce interfering colors. Formaldehyde reacts with phenol and represses color development. They have suggested reaction conditions that could eliminate these interferences.

A study of the behavior of various phenolic compounds in the 4-AAP method with or without distillation has been undertaken by Farino and co-workers [51]. For certain compounds such as phenol, o-cresol, o-chlorophenol, and 2,4-dichlorophenol the percentage of recovery did not change by distillation. But for compounds like resorcinol, and m-nitrophenol the recovery was less than 50% when distilled from aqueous solutions indicating that such compounds did not distill completely.

In a short communication, Goodwin and Marton [52] have described an automated distillation apparatus for the continuous flow determination of phenol in water. Since it has been noticed that different heating bath temperatures and changes in the concentration of the phosphoric acid gave different results with the 4-AAP method, their intention was to come up with a set of conditions that would give consistent results. Their system consisted of a heating rod and a distillation chamber located in a constant temperature bath. Chloride ions present in many samples containing phenols distill as hydrochloric acid and interfere with the determination by exceeding the capacity of the buffered hexacyanoferrate(III). A phosphate buffer of higher buffer

capacity that contains 78 g of trisodium phosphate (12 hydrate), 7.4 g of boric acid and 6.6 g of citric acid per liter of water was used to overcome this problem. A limit of detection of 5 μg l^{-1} has been reported. However, no mention is made of how they have determined it.

An automated flow injection system capable of analyzing up to 105 samples per hour has been described by Moller and Martin [53]. Potassium persulfate was used instead of potassium hexacyanoferrate(III) as the oxidant. This has resulted in a more stable baseline. A detection limit of 0.04 μg l^{-1} at a signal amplification factor of 5 on the spectrophotometer has been reported. Incorporating the chloroform extraction step as a part of the automated system, enabled them to analyze 50 samples per hour and improve the detection limit to 0.005 μg l^{-1}. But this enhancement of detectablility was found to be dependent on the phase ratios reached in the extraction module. The detection limit reported is based on the higher signal amplification factor and cannot be considered to be based on the actual concentration.

3-Methyl-2-Benzothiazolinone Hydrazone (MBTH) Method

This colorimetric method involves reacting phenol with 3-methyl-2-benzothiazolinone hydrazone in the presence of an oxidant (ceric ammonium sulfate) at an acidic pH. It is

TABLE II

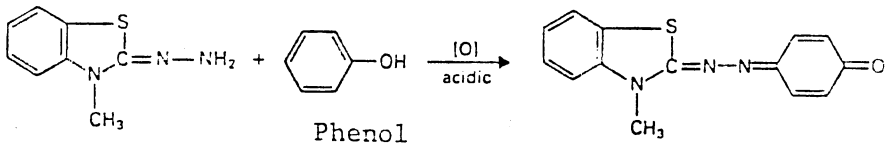
SUMMARY OF MODIFICATIONS AND IMPROVEMENTS TO 4-AAP METHOD

Modifications	Improvements	LOD*	Remarks	Reference
Extraction with n-butyl acetate or isoamyl acetate.	Quantitative extraction of phenol and cresols compared to about 80% extraction by chloroform.	0.5 μg l^{-1}		49
1.5 M NaOH used to back extract from the organic phase.	85% recovery compared to 15-20% using ammonia.			
Automation with extraction step incorporated.	Measurement of absorbance of organic phase improves sensitivity.			
5.0 ml of 1.0×10^{-3} M bismuth nitrate in concentrated HCl added and stored in glass bottles because phenols adsorb on the walls of the plastic bottles.	Water samples preserved for 2-3 weeks without loss from degradation or adsorption.		Adsorption of phenols on the walls of plastic bottles can be prevented.	
Distillation from a strongly acidic solution (10 ml of concentrated sulfuric acid per 500 ml).	Aromatic amines which interfere with phenol determination eliminated.	—	Large amouts of amines require double distillation.	50
Treatment with ammonium sulfate and NaOH followed by acidification with concentrated sulfuric acid to pH 4 and distillation.	Eliminates interference of formaldehyde.			

TABLE II (COTINUED)

Modifications	Improvements	LOD	Remarks	Reference
Automated distillation apparatus for continuous flow determination of phenol, heating coil and distillation chamber located in constant temperature bath.	Gives consistent results.	5 $\mu g\ l^{-1}$	Different heating bath temperatures gave different results.	52
124 ^{o}C and 10% phosphoric acid.	Almost 100% recovery for phenols of low boiling points.			
200 ^{o}C and concentrated phosphoric acid.	Better recovery for higher boiling substituted phenols.			
Automated flow injection manifold	105 samples per hour with good reproducibility		Signal amplification factor of 5 on the instrument	53
Chloroform extraction step incorporated to the automated FIA manifold	50 samples per hour		"	
$K_2S_2O_8$ used in place of $K_4Fe(CN)_6$ as oxidant	Stabler base line		Reproducibility problems with $K_4Fe(CN)_6$	
Steam distillation step with large excess of sodium chloride in an automated distillation apparatus	Improvement in the recovery of phenols Distillation time reduced to 2.5 minutes		Apparatus not described	
Automated system, distillation after color formation, extraction of dye into chloroform, two extractions	Higher sensitivity Sampling rate of 10 per hour Complete extraction of dye		Only one distillation step	45
Optimized conditions: pH: 10.5-11.0, temp.: 34 ^{o}C $K_2S_2O_8$ in place of $K_4Fe(CN)_6$	Stabler base line		Applicable to "clean" samples only	

capable of detecting para substituted phenols also. If the para position is occupied, the MBTH reagent will react at a free ortho position.



MBTH Phenol

A manual as well as an automated method is described by Friestad and co-workers [54]. The wavelength of maximum absorbance of the red-violet product formed by different phenolic compounds ranged from 460-595 nm. With the distillation step incorporated into the automated procedure, it was possible to process samples at the rate of 20 per hour with a relative standard deviation of 3.3% per 10 replicates. One problem that was encountered was the precipitation of ceric hydroxide when the slightly alkaline sample containing phenol and MBTH came in contact with the oxidant. The precipitate was dissolved by adding a buffer solution containing EDTA.

Goulden et al. [45] have determined phenol in ten samples of river water using both 4-AAP and MBTH methods and found no significant differences between the two methods. However, if the samples contained para substituted phenols, the results from the 4-AAP method should have been lower. No mention has been made of analyzing the water samples for

the presence of para substituted phenols. These researchers were able to avoid the precipitation of ceric hydroxide by adding EDTA to the reaction mixture very early in the system.

Morris E. Gales, Jr. [55] of the National Environmental Research Center, Cincinnati, OH, has evaluated the manual and automated MBTH methods to establish their applicability for the U. S. EPA to determine phenols in refinery waste water, surface water and domestic water. All absorbance readings were taken at an intermediate wavelength of 520 nm. His result showed that this method gave higher phenol concentrations when compared to the 4-AAP method. By increasing both the sample volume and the proportion of the reagent in the reaction mixture, a detection limit of 50 μg l^{-1} was achieved. Chloroform extraction following the development of color and subsequent determination gave a LOD of 1 μg l^{-1}. In the automated system in which the extraction step was not incorporated, a LOD of 1 μg l^{-1} was achieved by manipulating the flow rates.

Ultraviolet (uv) Methods

Direct method. Afghan et al. [49] have employed uv absorption spectroscopy to the determination of phenols after extraction with n-butyl acetate. Some of the phenols and cresols absorb in a narrow range between 285 and 295 nanometers. It is therefore possible to determine these

compounds at an intermediate wavelength of 292 nm. The authors have also reported the determination of trichlorophenol and pentachlorophenol by this approach for the first time. The limit of detection was 5 μg l^{-1}. However, this was achieved by using a cell of 10 cm pathlength.

Indirect method. Another uv spectrophotometric method for the determination of phenols has been reported by Bosch et al. [56]. This method is based on the measurement of the absorbance of the products of the reaction of phenols with iodine monobromide after extraction of the products with cyclohexane. They have determined the total phenol content of the "synthetic mixture" (60% phenol, 15% o-cresol, 15% m-cresol, and 10% p-cresol) recommended by Fountaine et al. [23] using 223 nm as the wavelength of maximum absorbance. The quantity of phenol was about 10-20% lower than originally present in the mixture. The reason for this disparity is not explained in the paper. One possible reason could be that they have used the wavelength corresponding to the products of phenol with iodine monobromide although the mixture contained o-, m-, and p-cresols. The maximum absorption of these compounds are at 214, 219, and 215 nm respectively.

Ratio method. The uv spectrum of a slightly acidic (ca. pH 6) solution of phenol shows a strong absorption band

at about 270 nm. When the solution is made basic (ca. pH 12) by the addition of NaOH, the band is shifted to about 291 nm. The new band is due to the formation of phenolate anion. This bathochromic shift is relatively independent of the amount of NaOH added.

Fountaine et al. [57] and Farino et al. [51] have used two hollow cathode lamps as sources of radiation: Lamp A - platinum, which is "pH sensitive" and Lamp B - chromium, which is "pH insensitive". With the sample in an acidic medium, the beam from Lamp A is blocked and the current from Lamp B is set to an arbitrary value. The shutter from Lamp A is then opened and the current to the lamp is increased or decreased so as to balance the intensities of the two wavelengths. When the sample is made basic, the beam from Lamp A is absorbed, proportional to the concentration of the phenolic compound present.

Fountaine et al. [57] reported the results of the determination of phenols using the uv-ratio method and compared the results with studies carried out with the 4-AAP methods. Instrumental parameters were optimized using ordinary phenol as the standard. Other phenols were determined using the calibration curve for phenol. The authors have used a "synthetic phenol" mixture consisting of 60% phenol, 15% o-cresol, 15% m-cresol and 10% p-cresol and determined the total phenol content using both methods. The phenol content determined by using the 4-AAP method was lower because it did not give a response for p-cresol.

Farino and co-workers [51] have also made a comparative study of the 4-AAP method with the uv-ratio method for the determination of various phenols without and with distillation. Their results were also similar to those reported by Fountaine and colleagues.

Difference method. Disinger and Manahan [58] described a method based on ultraviolet spectroscopy in which the difference between the absorbances of phenolic species at pH 12 and pH 7 was the basis for the quantitative determination of the analyte. Most phenolic compounds exhibit higher absorbance at pH 12 rather than at pH 7 due to the presence of phenolate anions. The authors have introduced a parameter,

$$a' = \frac{\text{phenol concentration } (\mu g\ ml^{-1})}{\Delta A}$$

$$\text{where, } \Delta A \text{ (absorbance)} = A_{pH12} - A_{pH7}$$

The average value of a' of all the phenols determined was calculated and using this value, they were able to calculate the 'total phenol' concentration of a mixture of phenols within a 6% error.

Higher Order Derivative Spectrophotometry

Higher order derivative spectrophotometry (HODS) is a very useful technique for the fine resolution of spectra. A

paper by Talsky [59] enumerates the application of HODS to environmental chemistry. Only phenol, aniline and pentachlorophenol were studied. Since the uv spectra of phenol and aniline overlap, the superimposed curves cannot be used for quantitative estimation. But the fourth and fifth order differentiation of the fundamental signal shows very good resolution. This system has the advantage that it can be applied to turbid water samples. Using this method, a detection limit for pentachlorophenol has been reported at 50 $\mu g\ l^{-1}$ with a mean deviation of 6.5%. Neither the method by which the limit of detection was calculated nor the number of replicates is mentioned in the paper.

A derivative uv-absorption spectrometer (DUVAS) described in an article by Hawthorne et al. [60] was designed and built at the Oak Ridge National Laboratory. It is a micro-computer controlled instrument which can provide a second-derivative spectrum by numerical differentiation of a digitized spectrum. The DUVAS was used to study phenols and methyl substituted phenols in real water samples from waste treatment plants and coal conversion facilities.

Raman spectroscopy

Application of laser excited resonance Raman spectroscopy to the determination of phenolic compounds is the subject of an article by Van Haverbeke and Herman [61]. A solution of the diazonium salt made from 4-nitroaniline

and sodium nitrite was mixed with a phenol solution. The 4-nitroaniline diazo dye formed was subjected to excitation by an argon gas laser and the resonance Raman spectra recorded. After subtracting the background spectrum, the authors were able to detect phenol at a concentration of 20 $\mu g\ l^{-1}$ based on a signal to noise ratio of 3. They were also able to obtain qualitative information by being able to identify o- and m-cresols from a mixture. Other derivatives of phenol formed from sulfanilic acid, 4-AAP and MBTH were also tested; but the results were not satisfactory. The application of this method is limited to those phenols that form colored derivatives with 4-nitroaniline.

Spectrofluorimetric Method

Afghan et al.'s paper [49] has also a brief description of the application of fluorescence spectrometry for the determination of phenols. The method is based on the measurement of the fluorescence intensity of an acidified sample at 305 nm when excited at 275 nm.

An attempt has been made by Chudyk and co-workers [62] to use a fiber optic probe coupled with uv laser-induced fluorescence for the remote detection of organic contaminants of ground water at a distance of up to 25 meters. The excitation wavelength was 266 nm. A detection limit of 10 $\mu g\ l^{-1}$ for phenol and 0.1 $\mu g\ l^{-1}$ for o-cresol has been reported. To arrive at this limit, they

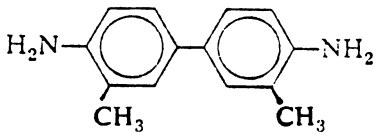
have used the concentration at which the signal is 1 standard deviation above the background. This can artificially lower the reported limit of detection. Much further work needs to be done to refine the technique and at the reported stage of development, it is more applicable as a detection technique rather than a method that could be employed for the quantitative determinaion of phenols.

Spectrophotometric Determination of Colored Products of Phenols with Other Reagents

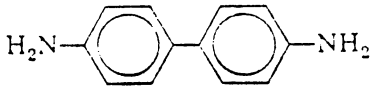
Spectral characteristics of the azo compounds formed by phenol and cresols resemble each other closely so that photometric determination of phenol in the presence of cresols is not possible without separating it from their mixture. The difference between the distribution coefficients of these compounds are also very small and complete separation is not possible by a single extraction. This problem has been addressed by Korenman and co-workers [63]. They proposed a method based on the different extractabilities of phenol and cresols by chloroform from aqueous solutions containing 4 mol/liter of $LiClO_4$. Using this approach, it was possible to increase the separation factor of phenol and cresols 3 to 4 times. Phenol that remained in the aqueous layer was reacted with $NaNO_2$ and a hydrochloric acid solution of sulfanilic

acid to form the azo dye. The absorbance of this product was monitored at 440 nm.

Two papers that describe the spectrophotometric determination of phenol via oxidative coupling has been published by Gupta and Verma [64] and by Baveja and Gupta [65]. In the first paper the coupling agent used was o-tolidine (4,4'-bi-o-toluidine) and benzidine was used in the work reported in the other paper.



o-Tolidine



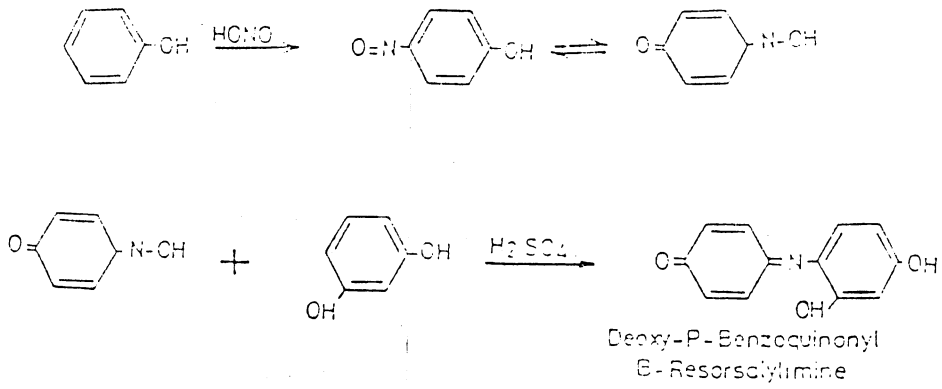
Benzidine

The dyes formed were stable for approximately 24 hours. A comparison of the results of phenol determination in river water with the results obtained using the 4-AAP method showed no significant difference.

A third paper by Gupta et al. [66] describes the determination of phenol in water and biological fluids by measuring the absorbance of the indophenol dye formed by the reaction of phenol with ammonia and N-chlorosuccinimide in the presence of sodium nitroprusside as a catalyst. The wavelength used in this case was 670 nm. The proposed method was compared with the conventional 4-AAP method by analyzing wastewater samples spiked with phenol. Recoveries in the range of 96.7 - 100.6% are reported. The results of these comparative studies made on polluted river or

wastewater are presented in the form of tables in their papers. No mention has been made whether a distillation step was carried out to purify and separate the phenols.

Hassan et al. [67] has proposed a colorimetric method in which resorcinol coupled to nitrosophenols has been used as the chromogenic agent. The reaction for phenol is given below:



The product has its maximum absorbance at 480 nm and phenol, o-, m-, and p-cresols have been studied. Whereas the 4-AAP method cannot determine p-cresol, this method can determine the compound in water samples.

A metal complex of the o-nitrosation product of chlorophenols is formed <u>in situ</u> when the chlorophenol, nitrite ions and a cobalt salt are made to react simultaneously. This reaction is the basis of a method reported by Zolotareva et al. [68] for the determination of certain chlorophenols. The absorption is measured at 575 nm. One limitation of this reaction is that nitrosation should occur at the ortho position with respect to the

hydroxyl group because only o-nitrosophenols are capable of undergoing complexation with metal ions such as Co^{2+}.

An improvement to the distillation process of phenols that can find wide applicability has been proposed by Norwitz et al. [69]. They have reported that the addition of a large amount of sodium chloride (15 g of NaCl to samples that contain about 4-20 mg of phenols) could improve the recovery of phenols during steam distillation. The main reason for this improved recovery is attributed to the sodium and chloride ions which in the aqueous solution are considered to be hydrated. This hydration tends to decrease the intermolecular hydrogen bonding of the phenolic compounds to water, thereby increasing the volatilization in steam. A summary of the observations on the various optical methods reviewed has been tabulated in Table III.

Gas Chromatographic Methos

Chromatographic methods in general and gas chromatography (GC) in particular have the advantage that under the proper conditions, the components of a mixture can be separated to a certain degree and identified. Coupled to a good detection system, GC methods can be highly sensitive. Because of these and other attractive features, GC has been used as a convenient and efficient method for the determination of phenols. The sensitivity of the technique for phenol determination can be further improved by incorporating one or more of the following features:

TABLE III

SUMMARY OF OPTICAL METHODS FOR THE DETERMINATION OF PHENOL

Method	Reagent	LOD*	Reproducibility (rsd,%)	Interferences	Advantages	Limitations	References
4-AAP - direct, manual	4-AAP, $K_3Fe(CN)_6$ $K_2S_2O_8$	-	1-3%	Turbidity, oxidizing, reducing agents, oils, tars, amines, formaldehyde, metals	Speed of determination, applicable to wide concentration range	All para substituted phenols not determined, loss during distillation, value reported only as phenol	41-44
4-AAP-extraction, automated	"	0.2 $\mu g\,l^{-1}$	1-5%	"	Better sensitivity	One or two extraction steps required	44,45 49-53
MBTH, manual	MBTH ceric ammonium sulfate, EDTA	1 $\mu g\ ml^{-1}$ (based on S/N=2)	1-8.3%	Ethanol, aldehydes, amines (removed by distillation)	Low blank readings, para substituted phenols can be determined	Uses phenol as standard. Value reported as phenol may not be true value	54
MBTH, automated distillation, extraction	"	1 $\mu g\ l^{-1}$ (based on S/N=2)	2-4%	"	"	"	45,54,55
UV-direct	-	5 $\mu g\ l^{-1}$ (10 cm path length)	-	-	Trichloro and pentachloro phenol determined	Uses one intermediate wavelength of 292 nm for all phenols	49

TABLE III (CONTINUED)

Method	Reagent	LOD*	Reproducibility (rsd, %)	Interferences	Advantages	Limitations	References
UV-indirect, product with iodine mono bromide	Iodine mono bromide, hydrazine, cyclohexane	1.1 μg l^{-1}	4.7-10.3% (10 replicates)	Most metal ions SO_4, NO_3, ClO_4, HCO_3	No distillation required, simpler, suitable for p-substituted and chlorophenols, cyclohexane extract stable	Total phenol concentration expressed as phenol	56
UV-ratio-batho-chromic shift	NaOH	5 μg l^{-1}	2-6.5%	Metal ions	Only one extraction necessary p-substituted phenols can be determined, only one simple reagent, very simple to use	Stability of lamp critical, less reproducibility with samples of low concentration, lacks selectivity between components of mixtures	51,57
UV-difference pH 7 and pH 12)	NaOH	1 μg ml^{-1}	6%	Coal gasification products	Large number of monhydric and polyhydric phenols analyzed. Possible to calculate a composite phenol concentration.	Polyhydric phenols have greater absorbance at pH 7 'Total phenol' value not very accurate	58

TABLE III (CONTINUED)

Method	Reagent	LOD*	Reproducibility (rsd, %)	Advantages	Limitations	Reference
HODS-2nd derivative	None	0.5 μg ml^{-1}	-	Better resolution of components of mixtures, turbid samples can be analyzed.	Low-noise signal, low pass filtering, signal averaging can improve signal to noise ratio.	60
HODS-4th & 5th derivatives	None	50 μg ml^{-1}	6.5%	All of the above. No sample pre-treatment necessary. Can be automated for continuous flow determinations.	All of the above.	59
Laser Excited Resonance Raman Spectroscopy	4-nitroaniline	20 μg l^{-1}	-	Possible to identify individual phenols.	Out of 126 phenolic compounds, only 28 produced colored derivatives with 4-nitroaniline, para substituted and polyhalogenated phenols do not form colored derivatives.	61

TABLE III (CONTINUED)

Method	Reagent	LOD*	Reproducibility (rsd, %)	Interferences	Advantages	Limitations	Reference
Spectrofluorimetric	-	0.1 $\mu g\ l^{-1}$	3-10%	Raman emission of water at 305 nm interferes with determination of low concentration of phenols	Solvent extraction by n-butyl acetate separated phenols before determination	Only phenol and O-cresol studied. Loss due to absorption and scattering. Cannot detect chlorophenols	49,62
Extraction, photometric determination of azo compound	$LiClO_4$, $NaNO_2$, sulfanilic acid	-	3.8%		Phenol can be determined in the presece of cresols. $LiClO_4$ capable of separating cresols during extraction	Only phenol and cresols studied.	63
Oxidative coupling	o-tolidine, benzidine, $K_2Cr_2O_7$, $K_4Fe(CN)_6$	-	1%	Metal ions, formaldehyde, sulfide, amines.	Comparable results with 4-AAP method Rapid. Dye stabler than antipyrine dye	Para substituted phenols cannot be determined	64,65
Indophenol dye	Ammonia, N-chlorosuccinimide, Sodium nitroprusside		"		Only 15 minutes required for color development, stable for 12 hours	"	66

TABLE III (CONTINUED)

Method	Reagent	LOD*	Reproducibility (rsd, %)	Advantages	Limitations	Reference
Benzoquinonyl β-resorsayl-imine	$NaNO_2$, resorcinol	4 $\mu g\ l^{-1}$	1-2%	para cresol can be determined	Only phenol and cresols studied	64
Complex of nitrosation product of phenol with a metal	$NaNO_2$, $CoCl_2$, $TBA^{@}_2$ in chloroform	0.08 $\mu g\ ml^{-1}$	-	Availability of common reagents	Only chlorophenols studied Chlorophenols should have at least one ortho position free	68

*LOD: Limit of detection
@TBA : tetrabutyl ammonium

preconcentration, derivatization, and automation. A brief description of each of these ancillary steps follows:

Preconcentration

Preconcentration of the phenols from water samples can be achieved by distillation [70], and extraction into organic solvents such as benzene [71,72] and dichloromethane [73,74]. Sometimes a single extraction may be sufficient to extract all the phenols. Often, more than one extraction using small amounts of solvents may be needed. Usually the extraction into organic solvent followed by the evaporation of extract results in loss of phenols because of their volatility. Direct GC determination without evaporation does not give satisfactory results, especially when only micro amounts of phenols are present in the sample. Addition of sodium chloride may help in the recovery [69]. Korenman et al. [75] have proposed a method in which several techniques are applied simultaneously for the preconcentration of phenols. An equimolecular mixture of n-butyl acetate and n-hexanol in the presence of 7 M camphor is used to extract phenols from 6 M NaCl solution. Even with phenol which is the most difficult component to extract, this method can achieve 90% recovery.

Recently, Caballero and co-workers [76] have applied solvent sublation using surfactants for concentrating phenol pollutants in 'synthetic sea water'. Solvent sublation is a

non-foaming flotation process by means of which the material adsorbed on the surface of gas bubbles is collected on a layer of immiscible liquid instead of in the form of a layer of foam over an aqueous phase.

Phenols have also been concentrated from water samples by using various solid sorbent materials. In this process phenols were first absorbed on to the solid support and then eluted using organic solvents. Some of the sorbents used include anion exchange [77] and cation exchange resins [78], porous polymer resins [78-82], and bonded phase silica materials [83-85]. Dichloromethane, diethyl ether, acetone, pyridine, chloroform, ethyl acetate, acetonitrile, and methanol are some of the solvents used to elute the phenols from the sorbents. A summary of this pre-concentration technique using sorbents is given in table IV.

Derivatization of Phenols

Phenols are usually converted into the corresponding ethers and esters by reacting them with suitable reagents. This type of derivatization leads to a decrease in the polarity of the phenol, thereby reducing the boiling point of the phenol and consequently the working temperature range. This in turn increases the number of stationary phases that can be used. Since the physical and chemical properties of the derivatives are different from the original phenols, better separation of peaks can be

TABLE IV

SUMMARY OF PRECONCENTRATION OF PHENOLS USING

SORBENTS PRIOR TO GC DETERMINATION

Sorbent			Eluent	Average recovery	Remarks	Reference
A-26	..	Anion exchange resin	4 M HCl followed by dichloromethane	100%	Quantitative recovery for phenol at pH 12.0-12.5	77
Sephron SE	..	Co-polymer of styrene and ethylene dimethacrylate	Methyl diethyl ether	83%	Reversed phase LC. m-Cresol can be separated from toluene	79
XAD-4	..	Polar macroreticular resin (Co-polymer of polystyrene and divinyl benzene)	Pyridine	85%	Distillation of pyridine-water azeotrope removes all water	80
XAD-7	..	"	Diethyl ether	67%	Higher % of recovery at pH 1	81
XAD-4/8	..	"	Acetone followed by $CHCl_3$	68%	o-Cresol has a higher % of recovery than phenol (46% recovery)	82
KU-23	..	Macroporous cation exchanger coated with n-pentyl acetate	1:1 mixture of 5% NaOH and 0.5% NaCl for phenol	90%	Less than 1 hour only required for the determination of phenols	78
Polisorb-1	..	Porous styrene/divinyl benzene co-polymer	5:1 mixture of water and acetone at pH 12 for ortho cresol, at pH 11 for p-cresol	-	o-Cresol and p-Cresol can be separated by changing the pH	78
C_2, C_4, C_8, C_{18}	..	Bonded phase silica	Ethyl acetate or acetonitrile Acetonitrile or methanol	-	pH 6-8 for neutral compounds pH 1-2 for acidic	83
C_{18}	..	Octadecyl modified	Acetone	85%	Good fractionation of all 11 priority pollutant phenols	84,85
CH	..	Bonded phase silica/ cyclohexyl modified	Acetonitrile or methanol	90%	CH-modified provided best recovery with NaCl as the 'salting out agent'.	83
DH	..	Diol modified	"			
CN	..	Cyanopropyl modified				

achieved. Some of the derivatives have strong electron-capture properties that increase the sensitivity of electron capture detector (ECD). Derivatization also has a similar effect on flame ionization detector (FID).

Some of the useful derivatives of phenols are: acetates [71,73,83,86-89], heptafluoro butyrates [77,89], pentafluoro benzyl ethers [73,90], pentafluoro benzoates [74,90], bis-trimethyl silyl acetamide derivatives [80], and bromo derivatives [70]. Most references describe a preconcentration step which, when coupled with the derivatization process, enhances the sensitivity of the method and the recovery of phenols from spiked samples. Distillation, extraction into organic solvents such as benzene and dichloromethane, and trace enrichment on modified silica gel are some of the preconcentration steps employed.

Acetates are usually prepared by the reaction of acetic anhydride with phenols in the presence of a basic substance such as K_2CO_3. The pH has to be controlled in the 9-10 range for the reaction to take place [84,89]. If the pH is too high, the acetic anhydride is destroyed; on the other hand, at a lower pH, the concentration of phenolate ions is less, resulting in reduced reaction rates. Potassium carbonate buffer gave a higher yield of the acetate derivative than potassium phosphate buffer[89]. Acetylation is a rapid method for derivatization of phenols which does not involve any hazardous reagent and gives

recoveries of over 85% of phenols from spiked water samples.

Lamparski and Nestrick [72] have used heptafluoro butyryl imidazole (HFBI) to derivatize phenol. Using a single extraction step with benzene, they preconcentrated the phenol from water samples. Although most of the phenol derivatives studied were stable, the derivatives of highly halogenated phenols and hydroxy phenols were stable for a period of less than 30 minutes only.

Pentafluoro benzyl ether and pentafluoro benzoate ester of chlorophenols have been prepared by Shang-Zhi and Duffield [90]. These derivatives give strong negative ion signals in mass spectrometry and by using electron capture-negative ion chemical ionization-gas chromatography-mass spectroscopy (EC-NICI-GC-MS), they have reported the lowest quantity determined as 0.1 pg (based on a S/N = 5). Though they report this as the detection limit, it is not expressed in concentration units as recommended by IUPAC. Pentafluoro benzyl ether [73] and pentafluoro benzoate [74] derivatives have been studied with electron capture detection and comparable results have been reported.

Though there are a number of advantages to derivatization, it has also some drawbacks. By preparing a derivative, an additional source of error is introduced. It is also not known whether all substituted phenols react quantitatively and form the derivative. Decomposition of unstable derivatives may affect the result. The different derivatives of phenol used in GC are summarized in Table V.

TABLE V

SUMMARY OF DERIVATIZATION OF PHENOLS IN GC METHODS

Derivative	Phenols studied	Pre-treatment	Solvent for extraction after derivatization	Stationary Phase	Average recovery of spiked sample	Remarks	Reference
Acetates	Phenols, chloro, nitro-phenols, cresols	Extracted into benzene	Hexane	OV-17-QF-1 OV-225 OV-101-OV-210	88%	Interferences from chlorinated degradation products	71
		Extracted into dichloro methane	"	Fused silica DB-1, DB-5	85%		73
		C_{18} cartridge	"	SE-30	85%		83
		-	Dichloromethane, water (1:1)	SP-1240-DA	97%	Reduced tailing of peaks Acetates of meta and para cresols could not be resolved	86,89
Heptafluoro butyrates (HFBI)[@]	Phenol, chloro phenols	Single extraction with benzene	Benzene	OV-1 OV-17	75%	Most derivatives stable Those of highly halogenated and hydroxy phenols stable for about 30 minutes	77
(HFBA)[ç]	also nitro phenols	-	Toluene	Fused silica OV-1	85%	Only 42% recovery for nitrophenols, acidified water samples at 60 °C gave higher yield, LOD 0.1 to 0.2 $\mu g l^{-1}$	89

@ HFBI : Heptafluorobutyryl imidazole
ç HFBA : Heptafluorobutyric acid

TABLE V (CONTINUED)

Derivative	Phenol studied	Pre-treatment	Solvent for extraction after derivatization	Stationary Phase	Average recovery of spiked sample	Remarks	Reference
Pentafluoro benzyl ethers	Chloro phenols	-	Hexane	OV-17 OV-101	-	0.1 pg reported as the lowest quantity determined (based on S/N=5)	90
	Also bromo phenols, phenol	Extracted into dichloro methane	"	fused silica DB-1, DB-5	70%	Less %recovery for mono chlorophenol and phenol	73
Pentafluoro benzoates	Chloro phenols	-	Hexane	OV-17 OV-101	-		90
	"	Extracted into dichloro methane	Hexane	fused silica coated with SE-34	80%	Pentachlorophenol not well resolved. Keeping low temperature in the final stage can overcome this problem	74
Bis-trimethylsilyl-acetamides	Phenol, cresols, chloro phenols	XAD-4 eluted with pyridine	Pyridine	SE-30 + OV-210, OV-1	85%	Distillation of pyridine-water azeotrope removes last traces of water	80
Bromo-derivatives	Phenol, cresols, 2,4,6-trichloro phenol	Distillation	Hexane	Carbowax-20M	90%	Salicylic acid interferes by forming bromoderivative 2,4,6-trichlorophenol passes through unchanged	70

Automation

Automation can be implemented at every stage of the operation for the determination of phenols by GC. A robotics system has been used by Hornbrook and Ode [91] for the microextraction of phenols from water. This system could be coupled to a gas chromatograph equipped with an automatic injector and a computerized data acquisition system. One such system has been described by Sporstol et al. [92] in which 73 organic pollutants in water have been determined using GC/MS combined with an automated search computer program. The computer program compares the retention time as well as the mass spectrum of compounds present with information of target compounds in the computer library. Limits of detection of 1 $\mu g\ l^{-1}$ or lower have been achieved with an average rsd of 3.2%.

Stationary Phases for GC

GC analysis of water samples have been carried out on both packed and capillary columns. Adsorbant materials such as silica gel [93], Tenax [94], and Tenax GC [77] have been occasionally used with limited success. Although these are thermally stable, their main disadvantage is that phenols undergo irreversible adsorption on such stationary phases [95]. This adsorption can be suppressed by modifying the surface with a small amount of liquid phase. It then falls into the type of separation technique known as

gas-liquid-solid chromatography.

Graphitized carbon black [96,97] has shown great promise as a solid sorbent for phenolic materials. Di Corcia et al. [96] have acid washed the material to eliminate basic carbonium oxygen complexes and sulfur. Acid washing has helped to eliminate chemisorption of p-nitrophenol on the column. The graphite surface was coated with trimesic acid (1,3,5-benzene tricarboxylic acid), Carbowax 20 M and Apiezon L by Mangani and co-workers [97]. Trimesic acid deactivates the alkaline active sites on the graphite surface, Carbowax 20 M increases the polarity and the non-polar Apiezon L lowers the retention time. These steps have improved the separation and quantification of all the eleven phenols included in the list of priority pollutants.

Non-polar, intermediate and polar liquid phases have been used in the majority of the papers reviewed. Fused silica, diatomite and polymer supports have been used to physically hold the liquid phases. On a non-polar stationary phase, the separation is primarily determined by the boiling point of the substance. The hydrogen bonding between the phenolic hydroxyl groups and the electronegative sites on the stationary phase is the most important factor that governs separation on the polar stationary phase [98]. The selectivity can be enhanced by increasing the polarity of the stationary phases.

Non-polar and semi-polar stationary phases are mostly

used when the phenols have been converted to a suitable derivative. Polar phases can be successfully used for the direct analysis of phenols without derivatization. Undesirable tailing of phenol peaks occurs at polar stationary phases. Addition of a small amount of phosphoric acid [88] can decrease the dissociation of phenols and can improve the separation efficiency and reduce tailing. A partial list of stationary phases used in GC is given in Table VI.

Identification and Determination of Phenols

Comparison of the retention data of phenols or their derivatives is the most common means of identifying phenols. Combination of GC with mass spectrometry (MS) is another avenue by which phenols are identified. Malissa, Jr. et al. [102] have used Fourier-transform infrared-spectrometry (FTIR) for the identification of phenols. This method is less sensitive compared to MS. Coupled with efficient enrichment of phenols from samples, new injection techniques and separation columns, FTIR can be an alternative technique for the identification of phenols.

The accuracy of the quantitative results from the determination of phenols depends on many factors. Losses during sample handling, extraction, derivatization and adsorption can lead to errors. The precision of the results

TABLE VI

STATIONARY PHASES USED FOR THE DETERMINATION OF PHENOLS BY GC

A. Adsorbants			Reference
Silica gel			93
Tenax	..	Porous polymer	94
Tenax GC	..	"	77
GCB	..	Graphitized carbon black, acid washed, coated with trimesic acid and polyethylene glycol	96
GCB	..	Graphitized carbon black coated with trimesic acid, Carbowax 20 M, and Apiezon L	97
B. Liquid Phases			
(i) Non-polar			
OV-1	..	Methyl silicone	73,80 93,99
OV-101	..	Dimethyl silicone	71
SE-30	..	" "	84,86,87 100,101
SE-34	..	" "	74
DB-1	..	" "	73
SE-54	..	Diphenyl dimethyl silicone	102
DB-5	..	" " "	92
(ii) Intermediate			
OV-17	..	Phenyl methyl silicone	71,72,77
(iii) Polar			
OV-225	..	Phenyl cyanopropyl methyl silicone	71
Carbowax 20M	..	Ethylene glycol	70,82,100
SP-1240-DA	..	Phosphoric acid deactivated polyester	76,88
OV-351	..	Ethylene glycol ester	101
FFAP	..	" " "	101
DEGS	..	" " "	100

depends on the purity of the standards and the experimental conditions.

The flame ionization detector and the electron capture detector are the two most widely used detectors in GC. Pre-concentration and derivatization of phenols can enhance the sensitivity of these detection methods. Especially when phenols are present only in trace amounts, accurate results cannot be obtained without these steps.

High Performance Liquid Chromatographic Methods

Another chromatographic technique that has found extensive use in the qualitative and quantitative determination of phenols in water samples is high performance liquid chromatography (HPLC). Because of the polarity and low vapour pressure of phenols, GC methods are not suitable for the direct analysis of water samples for the determination of phenols. Trace enrichment of phenols is often required followed by derivatization prior to gas chromatographic determination. These steps are often tedious and time consuming. Although preconcentration and/or derivatization can enhance the sensitivity of the HPLC method, direct determination of phenols from water samples is possible [94,103-107].

Preconcentration

The techniques used to preconcentrate phenols from water samples prior to GC and HPLC analyses are based on the same principles. Distillation [108], extraction into an organic solvent [109], cation exchange resin [109] and anion exchange resins [110-112] have been used occasionally. Adsorption on to various sorbent materials in packed columns or cartridges and subsequent desorption by suitable eluents is the most common method used for the trace enrichment of phenols. This step may be carried out off-line or on-line. A summary of this type of preconcentration of phenols using sorbents prior to their determination by HPLC is given in Table VII.

Stationary and Mobile Phases

Phenols being very polar substances are strongly adsorbed on polar stationary phases such as silica gel. One disadvantage of silica gel is the need to use a non-polar mobile phase that does not permit direct analysis of water samples. Lanin et al. [121] have described a normal phase HPLC method in which silica gel is used as the stationary phase and a mixture of hexane (non-polar) and 1-butanol (polar) as the mobile phase. Hexane being non-polar increases the retention time of the phenols and 1-butanol being polar decreases the retention time. One advantage of using such a mobile phase is that by varying the ratio of

TABLE VII

SUMMARY OF PRECONCENTRATION OF PHENOLS USING SORBENTS

PRIOR TO HPLC DETERMINATION

Sorbent	Eluent	Remarks	Reference
(a) Octadecyl modified silica			
LiChrosorb RP-18 u Bondpak C_{18}	Methanol:Water (60:40) containing phosphoric acid	Trace enrichment depends on particle size. Good results with 5-10 μm size	113
SepPak TM C_{18} cartridge	Acetonitrile:0.2 M sodium perchlorate with acetic acid (40:60)	Acetonitrile extract further concentrated using a stream of nitrogen after the addition of a small amount of NaOH which reduces loss of phenol.	108
LiChrosorb RP-18	Toluene:Hexane (80:20)	Addition of hexane to toluene improved the separation of the organic and aqueous phases.	114
(b) Styrene-divinyl benzene co-polymer			
PRP-1	Methanol:Water (80:20)	Mono- and dichlorophenols can be successfully concentrated. Comparable results with C_{18} column.	110 115-117
PLRP-1	Hexane:Toluene:Acetic acid (80:19:1)	Column was activated after use by passing methanol.	118
XAD-2/XAD-8	Acetonitrile:0.2 M sodium perchlorate (20:80) + 0.005 M acetate buffer	The eluent used could resolve the single cresol peak to two peaks corresponding to m- plus p-cresol and o-cresol.	119
XAD-2	Acetonitrile:Water (50:50)	Recoveries were higher with two connected precolumns	111

TABLE VII (CONTINUED)

Sorbent	Eluent	Remarks	Reference
(c) Cyclohexyl bonded phase			
BondElut	Methanol	Addition of sodium chloride prior to elution improved recovery of phenols	120
SepPak BondElut	Acetonitrile:Tri-sodium citrate buffer	pH 5 reduced the excessively long retention time of tetra and pentachlorophenols	107
(d) Carbonaceous materials			
PMS .. Pyrocarbon modified silica	Methanol:Water (60:40) containing phsphoric acid	PMS and PCMB are well suited for the trace enrichment of chlorophenols because of their high surface area. Particle size upto 30-50 µm can be used.	113
PCMB . Pyromodified carbon black	Acetonitrile:85% phosphoric acid (99:1)		
HTAC . High temperature treated activated carbon	"		

the non-polar and polar constituents, the retention time of the phenols can be increased or decreased.

CN- (cyano) [122] and NH_2- (amino) [123] bonded phases have been used in normal phase systems. Removal of all traces of water from the sample or the precolumn is very important in order to avoid changes in mobile phase composition as retention in normal-phase liquid chromatography (NPLC) is very sensitive to small changes in water content [98]. One or more extractions by organic solvents such as n-butyl acetate [121] and dichloromethane [123] were used to preconcentrate phenols from water samples. A reversed-phase pre-column packed with styrene divinylbenzene was used for on-line trace enrichment followed by desorption with n-hexane and toluene [118] to remove all traces of water.

Almost 90% of the papers reviewed have used reversed-phase chromatography with hydrocarbonaceous stationary phases. Octadecyl (C_{18}) modified silica was the preferred material for stationary phase. Methanol:water and acetonitrile:water of varying compositions were among the commonly encountered mobile phases. An acidic pH of 3-5 maintained by the addition of phosphoric acid or acetic acid or by a buffer containing phosphate or acetate helped to prevent the formation of phenolate ions and thus the tailing of the peaks. Both gradient elution and isocratic elution have been used.

Derivatization and Detection of Phenols

This step has been carried out either before the phenols have been separated on an HPLC column (pre-column derivatization) or after they have been separated (post-column derivatization). The 4-AAP derivatives of phenols were prepared by Blo et al. [122] by the usual procedure and extracted into chloroform. The chloroform extract was dried and then subjected to separation on the HPLC column. Bigley and Crob [120] on the other hand have used post-column derivatization to prepare 4-AAP derivatives of phenols. The absorbance due to the 4-AAP derivative in the visible region has been used for the detection and determination of phenols. Blo et al. [122] have used 480 nm as the wavelength of maximum absorbance. It has been noticed that actually there are two wavelengths (470nm and 509 nm) at which 4-AAP derivatives of phenols show maximum absorbance. Bigley and Crob [120] measured absorbance at both wavelengths and used the ratio of the absorbances to determine phenols. In addition to giving better quantitative results, this has also enhanced the selectivity.

Ratanathanawongs and Crouch [124] have used a post column reactor to prepare the azo derivatives of phenols. Yurchenko et al. [125] have resorted to pre-column derivatization procedure to prepare the azo dyes and then subjected them to ultrafiltration and HPLC separation prior

to spectrophotometric determination. Based on a signal to noise ratio of 2, a limit of detection of 32 μg l^{-1} has been reported by this method [125].

A recent journal article by Frei et al. [117] describes a process in which dansyl derivatives of chlorophenols have been prepared and separated on reverse-phase RP-18 column and subsequently decomposed by UV-irradiation using a post-column photochemical reactor to give degradation products with good fluorescence characteristics. A minimum detectable quantity of 200 pg in mass units (based on S/N = 2) for pentachlorophenol has been achieved by this method. In this paper, the authors point out that a solution of dansyl derivatives of phenols in methanol-water mixture, after irradiation, showed two emission maxima at 470 nm and 500 nm. The emission at 500 nm disappeared after several extractions using toluene. This is a very good method for the detection and determination of chlorophenols which otherwise cannot be detected by fluorescence measurement.

Ultraviolet (UV) Detection

This was the most common method of detection encountered among the papers under review. The UV spectra of phenols show an absorption band around 274 nm. Wavelengths of 270 nm and 280 nm are the most frequently used. Absorbance measurements were also carried out at 254 nm, the fixed wavelength used in most commercial UV

detectors. Buckman et al. [126] and Alarcon et al. [112] have carried out dual wavelength detection at 280 nm and 254 nm and used the ratio of the two measurements. Lower wavelengths of 220 nm [113,115] and even 202 nm [105] have been used especially for alkylphenols.

Fluorescence Detection

Fluorescence detection compared to UV detection is more sensitive and can give lower limits of detection. The excitation and emission wavelengths are ca.270 and 297 nm respectively. One disadvantage of fluorescence detection is that it cannot be directly applied to chlorophenols because chlorophenols cause fluorescence quenching. Werkhoven-Goewie and co-workers [115] have applied fluorescence detection to mono and dichlorophenols after photolytic dehalogenation using UV-irradiation. This method still cannot be applied to highly chlorinated phenols since they produce very little fluorescence even after photolytic degradation. The reason may be that these highly halogenated phenols on photolysis produce not only phenol but also other products that contain chlorine which may cause fluorescence quenching.

Electrochemical Detection

Phenols can be oxidized at carbon electrodes and this electrochemical behavior has been exploited for their

determination in a number of papers. Amperometric detection has been used in conjunction with HPLC in all the papers reviewed. Because of the higher sensitivity of this method, water samples can be analyzed directly without prior concentration. If a preconcentration step is employed, the sensitivity can be further increased. Though carbon paste electrodes are inert and can be easily replaced when fouled, they are not suitable in HPLC because the organic solvents (e.g. methanol or acetonitrile) used as mobile phases dissolve the oil used as the binder of the paste. The most common electrode material used was glassy carbon. Inactivation of the electrode surface due to the deposition of a polymeric film of the oxidation products of phenols is a common problem associated with electrochemical detection using glassy carbon or other carbon electrodes. Dual electrodes, each one poised at two different potentials, one at +0.250 V and the other at +0.650 V have been useful in excluding other electroactive species by oxidizing them at the lower potential [128]. Summaries of these detection methods are given in Tables VIII - X.

Electron Capture Detection

Though electron capture detection (ECD) is one of the commonly used methods of detection for phenols in GC, it has rarely been used in HPLC. Two recent papers by Maris et al. [114, 118] have demonstrated the potential of using both

TABLE VIII

HPLC OF PHENOLS WITH UV DETECTION

Direct/ Preconc- entration	Station- ary Phase	Mobile Phase	Wave- length (nm)	LOD/ MDQ	Remarks	Reference
Direct	C_{18}	Water containing 0.01 M phosphate buffer	280	10 μg l^{-1}	p-benzoquinone gave high response at 260 nm.	103
Direct	C18	Water-Acetonitrile, gradient elution	202	-	Most phenols absorb at higher wavelengths. Reports 96% recovery for phenol.	105
Direct	C_{18}	Isocratic elution, Mixture I:Acetonitrile-water-methanol-acetic acid (20:55:25:0.1) Mixture II:Acetonitrile-water-acetic acid (50:50:0.1) Mixture III: Acetonitrile-water-acetic acid (40:60:0.1)	254 280	2.5 ng	All eleven priority pollutant phenols can be separated by I 2,4,6-trichlorophenol and pentachlorophenol can be separated II and III. Sequential isocratic elution II (25 minutes) followed by I can also separate the priority pollutants.	126
Two step extraction with dichloromethane	C_{18}	Gradient elution, 30% acetonitrile in water to 80%, pH 3	254	-	Complete separation of all the phenols in 25 minutes. pH 3 prevents formation of phenolate ion and thus tailing.	109
Pre-column C_{18} and carbon materials	C_{18}	Gradient elution, Methanol-water with phosphoric acid	220 for chlorophenols	0.05-3 μg l^{-1}	Carbon materials found to be more efficient than C_{18} for pre-column concentration.	113

LOD : Limit of detection

MDQ : Minimum detectable quantity

TABLE VIII (CONTINUED)

Direct/ Preconc-entration	Station-ary Phase	Mobile Phase	Wave-length (nm)	LOD/ MDQ	Remarks	Reference
Micro pre-column	ODS	50% Acetonitrile	270	2 ng (S/N=4)	78-104% recovery	111
Micro pre-column	PRP-1	Methanol:water (80:20)	220	-	3- and 4-monochlorophenols not separated	115
Extraction by n-butyl after salting out	Silica gel	1-butanol with hexane	270	1 $\mu g\ l^{-1}$	Phenols strongly absorb on silica gel. By varying the composition of butanol and hexane, retention time of phenols can be changed.	
Pre-column	C_{18}	Acetonitrile:water Gradient elution to 80% Acetonitrile	280	250 $\mu g\ l^{-1}$	Except for phenol and nitro-phenols, recovery for all other priority pollutants were better	121
Preconc-entration on anionic exchanger	C_{18}	30 mM Ammonium Acetate (pH 5):Acetonitrile: Methanol (56:34:10)	254 280	0.1-0.8 $\mu g\ l^{-1}$	Buffer:acetonitrile(58:42) did not give complete separation of 2-nitrophenol and 2,4-dimethyl phenol.	127

LOD : Limit of detection

MDQ : Minimum detectable quantity

TABLE IX

HPLC OF PHENOLS WITH FLUORESCENCE DETECTION

Direct/ Preconc-entration	Station-ary Phase	Mobile Phase	Wave-length	LOD	Remarks	Reference
Micro pre-column	PRP-1	Methanol:Water (80:20)	ex: 271 nm em: 290 nm	-	Post column photoconversion of lower chlorinated phenols at neutral or acidic pH	115
Direct	C_{18}	Acetonitrile: Water (40:60) pH 4.6	ex: 266 nm em: 300 nm	-	Limited to hydroxy benzenes only	94
Precolumn	C_{18}	Acetate buffer: methanol (50:50)	ex: 271 nm em: 297 nm	10 ngl^{-1} (pptr) (S/N=3)	Pre-column concentration improved recovery and limit of detection	111
Extraction	Amino silica	Hexane:isopropyl alcohol (98.5:1.5)	ex: 277 nm em: 300 nm	0.1 to 0.2 μgl^{-1}	70-75% recovery for water samples spiked with 1-3 μgl^{-1} of phenols	123
Dansyl derivative	RP-18	Methanol:Water (85:15) contain-ing phosphate	ex: 330 nm em: 470 nm	-	Higher chlorinated phenols also can be detected after UV-irradiation of dansyl derivatives	117

ex: excitation wavelength

em: emission wavelength

TABLE X

HPLC OF PHENOLS WITH AMPEROMETRIC DETECTION

Direct/ Preconc-entration	Station-ary Phase	Mobile Phase	Electrode/ E_{app} vs Ag/AgCl	LOD/ MDQ	Remarks	Reference
Direct	Cation exchange resin	Acetonitrile-water containing 0.025M sulfuric acid	Carbon black/ polyethylene tubular electrode +1.20 V	0.2 to 0.7 $\mu g\ l^{-1}$	Acid suppresses phenol ionization, provides supporting electrolyte	104
Direct	C_{18}	Acetonitrile:water (30:70) containing 0.05M phosphate (pH 4.2)	Kel-F-Graphite composite electrode, +1.27 V	3.8 pg	Optimum applied potential depends on the pH	106
Direct/ Off-line trace enrich-ment	ODS	Acetonitrile with 0.2M perchlorate and trisodium citrate (pH 5)	Glassy carbon dual electrode +0.850 V and +0.900 V	120 ng l^{-1}	Dual electrodes poised at two different potentials, current ratios provide additional selectivity	107
On-line/ off-line pre-conc-entration	RP-18	Methanol with 0.02M KNO_3	Glassy carbon +1.00 V	-	85% recovery for phenols Loss of sensitivity due to electrode fouling, several calibrations every day.	116
Distill-ation. C_{18} column	C_{18}	Acetonitrile: perchlorate (40:60) + Sodium Citrate and acetic acid	Glassy carbon + 0.950 V and + 0.850 V	-	Electrochemical responses of m- and p- cresols similar at 0.95 V, but different at 0.85 V. Using this inform-ation their concentrations can be calculated.	108
Pre-column	Styrene-DVB pol-ymer	Acetonitrile:water (25:75) with 10 mM phosphate (pH 9.2)	Dual electrode + 0.250 V and + 0.650 V	0.034 $\mu g\ l^{-1}$	Other electroactive species excluded at the lower potential.	128
Pre-column	C_{18}	Acetonitrile:0.2 M perchlorate (20:80) + 0.005 M acetate buffer (pH 5)	Dual electrode parallel: + 1.0 & 0.65 V series: + 1.0 & -0.20 V	2 ng	Parallel electrodes used to discriminate groups of compounds. Oxidation products formed reduced at lower potential.	119

E_{app} : Applied potential, LOD : Limit of detection, MDQ : Minimum detectable quantity

reverse-phase and normal-phase HPLC with ECD. Comparable detection limits of 0.4-10 μg l^{-1} for chlorophenols have been achieved with a precolumn enrichment step also incorporated into the system.

Miscellaneous Methods

The major bulk of the techniques employed for the determination of phenols in water fall into the optical or the chromatographic methods already reviewed. A few other methods that cannot be classified into any one of these categories have been included in this section. The most common among these miscellaneous methods are: electrochemical methods, kinetic methods, potentiometric methods and enzymatic methods.

Electrochemical Methods

A paper by McCrory-Joy [129] describes the electroanalytical chemistry of chlorophenols at a glassy carbon electrode. He has carried out cyclic voltammetric studies to investigate the electrochemistry of chlorophenols and used differential pulse voltammetry to determine the phenols in a methanol-0.07 M sulfuric acid medium. The detection limits of the various trichloropehnols and pentachlorophenol varied from 0.5-3.4 μg ml^{-1}.

In a recent paper, Hernández et al. [130] have used a carbon paste electrode modified with sepiolite using

differential pulse voltammetry for the determination of phenol. A preconcentration step was performed by placing the electrode in a cell containing the phenol solution for a fixed period of time with constant sirring at pH 1.5 under open circuit conditions. The electrode was washed with water and then placed in an electrolytic cell and the measurement was carried out by recording the differential pulse voltammogram. A detection limit of 0.1 μg ml^{-1} has been reported for phenol. No analysis of water samples was included in this study; but the method was applied to the determination of phenol in carbonated beverages. The chemical composition of sepiolite was never mentioned in the paper nor was the source from which it was procured indicated. Sepiolite is a soft clay material whose composition is $Mg_3Si_4O_{10}(OH)_2 \cdot 4H_2O$.

Cañete et al. [131] have explored the possibiliy of using fast scan cyclic voltammetry in conjunction with flow injection analysis for the determination of phenolic compounds. Conventional current versus potential as well as derivative curves were recorded. Using the derivative mode, a limit of detection of 20 μg l^{-1} has been reported. The authors have also attempted simultaneous determinations of binary mixtures. Since the half-wave potentials of phenol and 2,4-dichlorophenol were 577 mV and 651 mV (vs Ag/AgCl) respectively and there was overlap, the resolution of a mixture containing both was difficult to achieve. By establishing simultaneous equations with two unknowns and

solving them, it was possible to determine the concentrations of the two components.

Kinetic Methods

Sand and Huber [132] have applied differential constant current potentiometry to the determination of 2,6-dichlorophenol by bromination. In differential constant current potentiometry, the difference in potential between two electrodes held at constant current is monitored. The experiment is carried out under stirring conditions and a change in potential occurs when the concentration of the electroactive species changes near the electrode. The potential versus time curve has a peak shape when an electroactive species undergoes depletion by homogeneous reaction in solution. The width of the peak is a measure of the reaction rate. Plots of reciprocal time in minutes versus the concentration of 2,6-dichlorophenols at different potentials are linear, with the one at 600 mV showing maximum sensitivity. The limit of detection of this method for the determination of the analyte has not been reported. But the authors expect the lowest concentration that can be detected by this method to be about 10^{-4} M corresponding to about 16 μg ml^{-1}.

A fixed time method for the kinetic determination of phenols and chlorophenols has been proposed by Sherman et al. [133]. Phenols were oxidized to quinols and quinones by

metaperiodate in a constant temperature bath maintained at a temperature of 50 ± 1 ^{o}C. The reaction was carried out in a methanol-acetic acid medium and was allowed to proceed for a pre-determined period of time for each phenol. The reaction mixture was then cooled and its absorbance was measured at 462 nm. The concentration of the phenol can be calculated from the equation

$$X = K\ a\ C\ t$$

where, X is the absorbance measured, K is the analyte rate coefficient, a is an experimental constant and t is the predetermined time.

A more detailed study of the same fixed-time kinetic method for the determination of phenols and chlorophenols has been undertaken by Buckman et al. [134]. A reaction time of 120 minutes was used throughout the study. The absorption spectra were recorded over the 300-320 nm range and whenever appropriate, was corrected for methanol absorption in the 305-313 nm range. However, the wavelength at which the absorbance readings were taken for the purpose of calculation is not indicated in the paper. Amount of methanol, metaperiodate concentration and acetic acid concentration were optimized. The limit of detection for this method is only 50 μg l^{-1} and consequently this method has only limited applicability to the determination of phenols in natural waters.

A recent journal article by Martinez-Lozano et al. [135] describes a photokinetic method for the determination

of trace amounts of phenol and acetaminophen. The photochemical reaction of methylviologen (MV^{2+})-EDTA-acridine yellow (AY) is strongly inhibited by the presence of even small amounts of these compounds.

The authors have derived an equation in the form:

$$\frac{Vo}{Vx} = 1 + K\,[\text{Inhibitor}]$$

where, Vo = reaction rate without inhibitor

Vx = reaction rate with inhibitor

K = overall inhibition constant

The value of K for phenol has been found to be 3.3×10^{3} liter mol^{-1}.

Calibration curves were constructed by plotting the analyte concentration versus t_x/t_o, where t_x is the time required for the sample to reach a pre-fixed value of absorbance (0.8 A) and t_o is the time required for an analyte-free sample to reach the same value. The detection limit of phenol reported is 6.2×10^{-6} M (0.6 μg ml^{-1}). This method has been applied to aqueous solutions of pharmaceutical preparations containing phenol and acetaminophen. Extension of the method to water samples must be possible though interferences from metal ions have to be suppressed by the addition of EDTA.

Ring Oven Method

Chlorophenols after *in situ* derivatization with 3-methyl-2-benzothiazolinone hydrazone (MBTH) has been

determined using ring oven technique by Buckman and co-workers [136]. Using a series of standard solutions of phenol at the μg l^{-1} level, the MBTH derivatives were produced in situ on a filter paper and their color developed in the ring oven operated at a temperature of 100 ^{o}C. The derivative of the unknown phenol solution was also prepared in a similar manner and by comparing the color intensities of the ring with those of the standard solutions, its concentration was determined using the method of Weisz [137]. The authors have also tried in situ extraction of the MBTH derivative using chloroform and reported recoveries close to 100%. Using in situ solvent extraction, they were also able to separately quantify each component of a binary mixture of phenols. Since the method is based on visual comparison of the color intensities of rings produced on filter papers, it can be considered only as a semiquantitative method and is not expected to give very accurate results. A "densitometer" similar to the one used to quantify TLC plates may also be used.

Potentiometric Titrations

In the plot of the pH versus the titrant volume of a weak acid such as phenol in water, the inflection at the equivalence point is not sharp. In order to overcome this problem, Kamakura [138] used plots of pH versus the

logarithm of the titrant volume for the determination of phenol in water. The titrant used was carbon dioxide free sodium hydroxide. The plot had a very good inflection between the two linear portions and the equivalence point could be easily determined by the graphical method. Phenol solutions in the concentration range of 1×10^{-3} M to 7.7×10^{-3} M were titrated by this method. Often phenols are present in water samples at much lower concentrations and therefore, this method has little application to real samples.

Two papers by Mohandas and Indrasenan deal with potentiometric titrations of various substances including phenol using different oxidants, N-bromophthalidimide (NBP) [139] and N-bromosaccharin (NBS) [140]. The titrations were carried out in aqueous acetic acid medium. The general half-cell reaction can be represented as:

$$RNBr + H^+ + 2\ e \longrightarrow RNH + Br^-$$

where, RNBr stands for the oxidant.

The bromination of phenol required added potassium bromide (0.5 g) since the actual oxidant was the bromine produced <u>in situ</u> by the reaction of NBP or NBS with the potassium bromide. The potential jumps were very sharp and a steady potential was attained very quickly. Phenol was titrated in the 0.04-0.08 mM range. The authors state that there was no need to add any acid (perchloric or hydrochloric acid) to get a sharp potential break. Actually the medium in which they were titrating phenol contained

acetic acid.

Enzymatic Methods

The catalytic action of enzyme tyrosinase on phenols has been described in Chapter I. The quinones produced by this reaction can be easily reduced electrochemically. Schiller et al. [34] immobilized tyrosinase by the entrapment of the enzyme on an acrylamide gel cast around a basket-woven platinum screen. They used this electrochemical system to determine phenol concentration in aqueous solutions by measuring the potential difference created when the quinone formed by the oxidation of phenol was reduced by the hexacyanoferrate(II). A limit of detection of 40 $\mu g\ ml^{-1}$ was achieved .

By supporting the enzyme tyrosinase on a nylon membrane and using it as part of a graphite foil electrode, Hall et al. [141] were able to detect p-cresol in chloroform by the electrochemical reduction of its oxidation product 4-methyl-1,2-benzoquinone. The electrode was poised at -275 mV vs SCE and the current generated was recorded. They were able to detect p-cresol at a concentration of 1 µM corresponding to about 0.11 $\mu g\ ml^{-1}$. Before each set of determinations, the electrode has to be dried for about 30 minutes and a small quantity of phosphate buffer had to be added to the sides of the electrode to maintain the hydration of the enzyme. The time consuming process of

conditioning the electrode with the added disadvantage that the reaction is carried out in chloroform precludes the application of this method to the analysis of water samples.

Bonakdar et al. [142] have incorporated the enzyme tyrosinase together with poly-4-vinyl pyridine (PVP)-immobilized hexacyanoferrate(II) as a redox mediator in a carbon paste electrode and used this as an amperometric sensor for the continuous-flow determination of phenol in aqueous solutions. At the applied potential of -0.200 V (vs Ag/AgCl), the o-benzoquinone produced by the catalytic oxidation of phenol is reduced to 1,2-dihydroxybenzene with the concomitant oxidation of hexacyanoferrate(II) to hexacyanoferrate(III). With the injection of a sample of phenol, the current produced by the above reaction is recorded as a flow injection peak. A limit of detection of 14 $\mu g\ l^{-1}$ has been achieved by this method. One drawback of this method is that the enzyme slowly loses activity with time and also it cannot be reused.

Edible mushrooms have been found to contain sufficient tyrosinase naturally immobilized [143]. Bonakdar et al.[142] have also attempted to use fresh mushrooms along with the hexacyanoferrate mediator in a carbon paste electrode for the detection of phenols. The mushrooms needed repeated washings with the supporting electrolyte to be conditioned for use. A detection limit of 0.16 $\mu g\ ml^{-1}$ has been reported. The authors have also tested a dual-electrode thin-layer cell with one of the wells packed

with fresh mushroom and the other packed with the carbon paste modified with the PVP-immobilized hexacyanoferrate mediator. That part of the cell with the mushroom acted as an enzyme reactor and the other part operated as the amperometric sensor. This configuration gave reasonably good response for phenol and the response depended on the mushroom sample used. No real water samples have been analyzed by this method.

Conclusions

Among the analytical techniques for the determination of phenols in water, the 4-AAP method is the most widely used. This method is sensitive especially when used in conjunction with chloroform extraction to preconcentrate phenols. Only the 'total phenol' content can be determined which does not include most of the para substituted phenols that do not react with 4-AAP. This method also does not provide information on the composition of a mixture of phenols. During the distillation step, which is often carried out prior to determination, some of the phenols may be oxidized which may also lead to lower results.

The other spectroscopic methods, such as Raman and IR spectroscopy and mass spectrometry, provide data useful in the elucidation of the structure of phenols and can be used in the identification of individual phenols. Fluorescence detection, though more sensitive than the other optical

methods cannot be directly applied to chlorophenols.

Photometric determination of the other colored derivatives of phenols give results comparable to 4-AAP method. Some of them have the advantage that para substituted phenols can also be determined. But derivatization involves the use of certain reagents which are toxic or explosive. UV methods are simple to use and the higher order derivative techniques can be successfully applied to the determination of phenols in turbid water samples without pre-treatment.

When phenols are present in complex samples such as industrial effluents, a separation step is often required to isolate phenols from other constituents. Chromatographic techniques provide one of the best separation in such cases. Most phenols are strongly polar and more complex phenols are less volatile which complicate their determination using GC methods especially when these compounds are present only in trace amounts. Pre-concentration and/or derivatization steps are required to improve the sensitivity of GC determination. Direct aqueous injections are not generally done in GC because water strips the liquid phase and decreases column life.

HPLC has the advantage that the chemical composition of the stationary and mobile phases can be varied to meet specific needs and by using either normal-phase or reverse-phase techniques, better separation of phenols can be achieved. Moreover, unlike GC, HPLC is not limited to

volatile substances and can be used for direct determination of aqueous samples containing phenols. Coupled to a good detection method, HPLC can be highly sensitive.

Electrochemical detection methods have the advantage that water samples can be directly analyzed even when they are colored or turbid. Because of its high sensitivity, a preconcentration step is not warranted. Kinetic methods can be applied for the trace determination of individual phenolic compounds. Analysis of binary mixtures requires setting up of simultaneous equations and solving them.

Each of these methods for the determination of phenolic species in water samples has its own advantages or disadvantages. The choice of a particular method has to be based on such considerations as the complexity of the sample, the concentration at which phenols are present in the sample, whether direct anlaysis is required or preconcentration/derivatization steps are to be carried out. Depending on the availability of instrumentation and reagents, a method can be chosen to suit individual needs.

CHAPTER IV

STUDIES ON IMMOBILIZED TYROSINASE

Introduction

According to the literaure reviewed in Chapter II, immobilization of tyrosinase has been accomplished by various methods. Although tyrosinase has been immobilized successfully by covalent coupling, physical entrapment of the enzyme within a polymer matrix or membrane has been the most common method employed. Inactivation of the enzyme and leakage leading to loss of activity with time are among the major disadvantages of this method. Covalent attachment of enzymes to water insoluble matrices is the most widespread approach to enzyme immobilization. Although covalent attachment involves more complicated and often less mild conditions than physical entrapment methods, it results in stable immobilized enzyme preparations which do not leach into solution.

The availability of reactive residues in the protein is an important factor to consider while selecting a reaction pathway to be used for covalent attachment. The enzyme tyrosinase contains about 5.2% L-lysine and 4.2% L-tyrosine [144] which are among the most convenient residues that can be used for immobilization [145]. The ε-amino group of

L-lysine and the phenolic -OH group of the L-tyrosine are excellent for diazo coupling with a diazonium salt.

Controlled-pore glass (CPG) was chosen as the support matrix to be used for the immobilization of the enzyme tyrosinase. CPG is a microporous high-silica glass prepared from borosilicate glass. This type of glass undergoes a phase separation by heat treatment (500-700 ^{o}C), and subsequent acid leaching of the borate component leaves a porous structure of very high silica content [146]. CPG has been widely used as a support material for enzyme immobilization. It possesses excellent mechanical strength, thermal stablity and is resistant to organic solvents; all good characteristics of an inert support. Being porous, CPG has a high surface area per unit weight which allows for high enzyme loading.

Direct coupling of the enzyme to the CPG is not possible since it does not contain the diazonium salt group. A spacer with an aromatic amine group that could be further diazotized was attached to the CPG by a method similar to the one developed by Marshall and Mottola for the synthesis of silica immobilized 8-quinolinol [147].

Experimental Methods and Procedures

Reagents, Solutions, and Materials

All chemicals were of "Analytical Reagent" grade, except when noted. The water used for the preparation of

solutions was deionized and was further purified by distillation in an all-borosilicate glass still with a quartz immersion heater (Wheaton Instuments, Millville, NJ). (Aminophenyl) trimethoxy silane (Petrarch Systems, Briston, PA) was obtained as mixed isomers and stored under refrigeration. Tyrosinase from mushroom and L-tyrosine were obtained from Sigma Chemical Co. (St. Louis, MO). Controlled-pore glass (CPG 0500) of mean pore diameter 515 A and mesh size 200/400 was procured from Electronucleonics, Inc. (Fairfield, NJ). 0.10 M phosphate buffer was prepared by dissolving appropriate amounts of KH_2PO_4, potassium hydrogen phosphate (monobasic) crystals in deionized distilled water. The pH was adjusted with 6.0 M NaOH.

Apparatus

A Perkin-Elmer Lambda 3840 UV/VIS linear diode array spectrophotometer operated by a Perkin-Elmer 7300 computer (Perkin-Elmer Inc., Nowalk, CT) and an Integral Data Systems P-132 printer (Integral Data Systems, Inc., Milford, NH) was used for the collection, manipulation and output of all spectral and absorptiometric measurements. Adjustment of pH was made with an Orion Research Model 601A pH meter (Orion Research, Cambridge, MA) equipped with an epoxy-body combination electrode (Sensorex, Westminster, CA).

Procedure for Silylation of
CPG Using Toluene

About 2.0 g of CPG was dried in an oven at 120 ^{o}C overnight. 20 ml of a 10% solution of (aminophenyl) trimethoxy silane in dry toluene were added to the CPG and the reaction mixture was refluxed for about 4 hours. The aryl amine-CPG (AA-CPG) thus formed was filtered under vacuum, rinsed with toluene, and dried overnight in an oven at 80 ^{o}C.

Procedure for Diazotization of the
Aryl Amine-CPG

100 ml of 2% $NaNO_2$ in 2.0 M HCl were added to the AA-CPG at about 0 ^{o}C, and the reaction was allowed to continue for 30 minutes. This reaction results in the formation of the diazonium salt. The product was quickly filtered, washed with three 15 ml portions of cold 0.10 M potassium phosphate monobasic buffer of pH 6.5. It was then transferred to a 250 ml beaker.

Procedure for Immobilization

About 5.0 ml of tyrosinase dissolved in the phosphate buffer corresponding to about 250 units of enzyme activity were added to the beaker containing the diazotized AA-CPG. The contents of the beaker were kept in the refrigerator at

about 4 ^{o}C for 45 hours for the reaction to continue. The CPG-immobilized tyrosinase was filtered under vacuum and washed repeatedly with the buffer to remove any free enzyme. When almost dry, it was divided into different portions, weighed and stored in the refrigerator in a small quantity of the buffer.

When another batch of CPG-immobilized tyrosinase was prepared, the final product after filtration was placed inside a desiccator and the desiccator was kept in the freezer compartment of the refrigerator. In this way, a lyophilized form of the immobilized preparation was stored for future use.

Determination of Activity Using L-Tyrosine

This procedure was adapted from the Worthington Enzyme Manual [148]. Air was bubbled into a 1.0 x 10^{-3} M solution of L-tyrosine for about 10 minutes. A sample of the CPG-immobilized enzyme stored in the buffer was taken in a quartz cuvette. About 2 ml of the phosphate buffer (pH 6.5) were added to the cuvette and the background was scanned using the Perkin Elmer Lambda Array Spectrophotometer. 1.0 ml of aerated tyrosine was added to the cuvette which gave a final concenration of 3.3 x 10^{-4} M of the substrate. The absorbance at 277 nm was scanned every 2 minutes for 20 minutes. Between scans, the

cuvette was taken out and shaken for about 1 minute. A typical plot that shows the increase in absorbance with time for L-tyrosine by the action of immobilized tyrosinase is given in Figure 1.

Determination of Activity Using Phenol

The experimental procedure was similar to the one used for the determination of activity using tyrosine. 1.0 ml each of 1.0×10^{-3} M phenol, 0.10 M buffer, and 1.0×10^{-2} M potassium hexacyanoferrate(II) were transferred to a cuvette containing the CPG-immobilized tyrosinase and change in absorbance was monitored at 420 nm for 20 minutes.

Determination of Activity of Mushroom

Tyrosinase used in this study was obtained as a purified preparation from mushroom. The edible variety of mushroom (*agaricus bisporus*) is readily available and is a source of naturally immobilized tyrosinase. The tyrosinase activities of different varieties of mushrooms were investigated in a similar manner using phenol as substrate.

Results and Discussion

The various steps involved in the immobilization of tyrosinase on CPG are as follows:

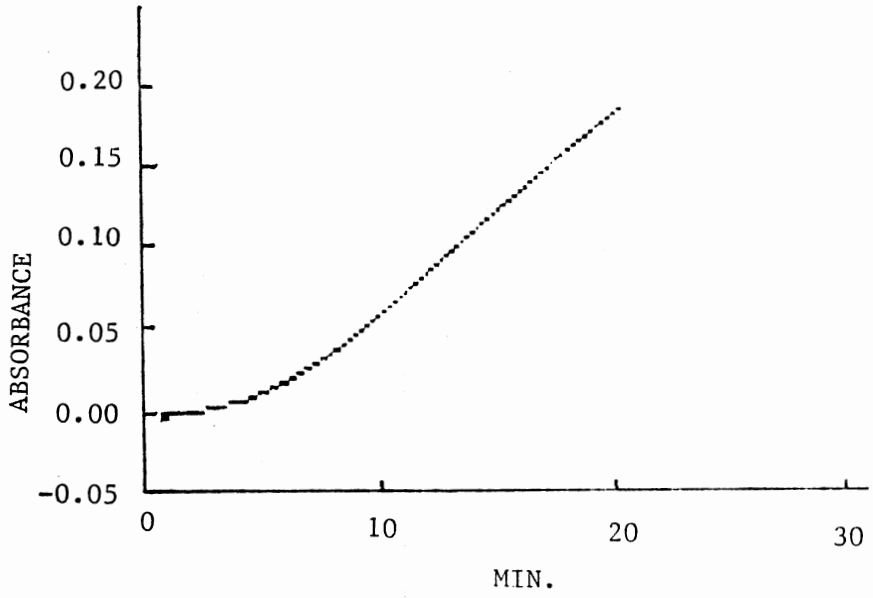
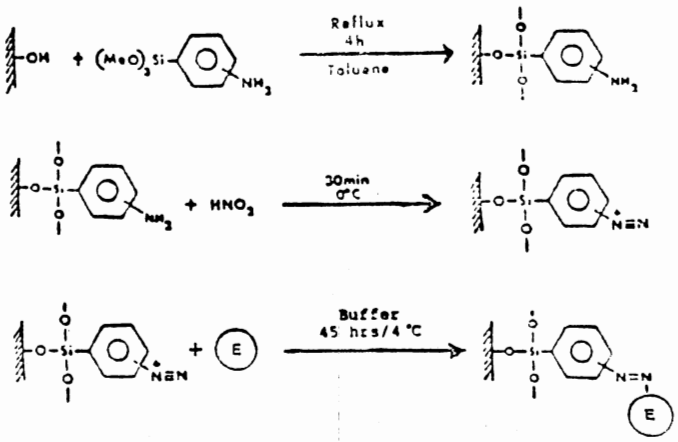
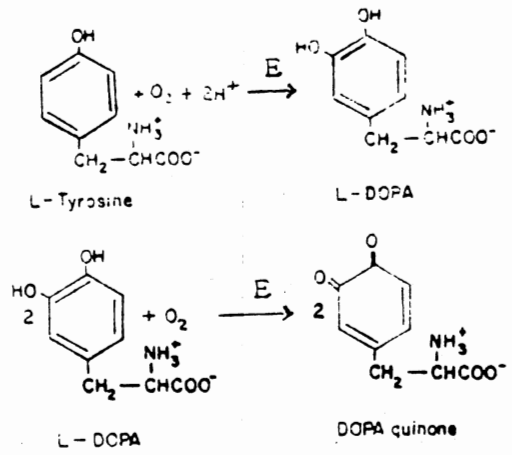


Figure 1. Increase in absorbance with time for L-tyrosine by the action of immobilized tyrosinase



E = Enzyme

In the presence of oxygen, tyrosinase oxidizes L-tyrosine to dihydroxy phenyl alanine (DOPA) first and then to DOPA quinone as shown by the following equations:



E = Enzyme

The formation of DOPA quinone is accompanied by an increase in absorbance at about 280 nm which can be monitored spectrophotometrically. An initial lag of

approximately 2-3 minutes was noticed with this reaction. After that there was a linear increase in the absorbance.

In order to check the reusability of the CPG-immobilized enzyme, it was washed repeatedly with the buffer after monitoring the activity with tyrosine. The activity was determined again and this process was repeated in order to ascertain the retention of activity with time. Whenever necesary and especially when it had to be stored overnight, the CPG-immobilized tyrosinase was kept in the refrigerator in a vial containing a small amount of the buffer. The percentage of activity lost on each subsequent use was computed by comparing the change in absorbance values (Δ A) with that obtained when the immobilized preparation was used the first time. The change in absorbance values and the percentage of activity lost when a sample of immobilized preparation was repeatedly used with 3.3×10^{-4} M tyrosine is given in Table XI.

Tyrosinase becomes progressively inactive by repeated use with tyrosine; as it is evident from Table XI, about 3/4ths of the original activity of the enzyme has been lost when used seven times. This "reaction inactivation" has been attributed to covalent attachment of the substrate or the product(s) of the reaction to the enzyme. The suggested explanation for this effect is the nucleophilic attack of lysine amino groups of the enzyme on the quinone product yielding a covalent adduct which blocks the active sites [149]

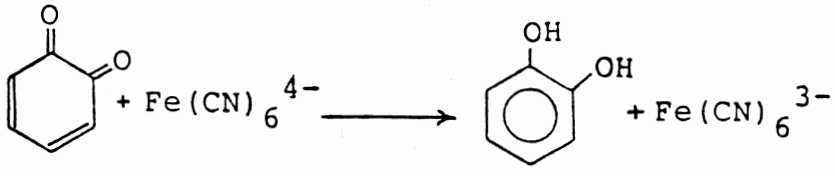
TABLE XI

RESPONSE OF CPG-IMMOBILIZED TYROSINASE TO TYROSINE

Use	Δ A 4-20 min	% of activity lost compared to 1st use
1	0.149	-
2	0.138	7.6
3	0.077	48.6
4	0.058	61.4
5	0.052	65.3
6	0.049	67.1
7.	0.040	73.4

To find out whether this is a competitive inhibition, 1.0×10^{-2} M solution of phenol which also is a substrate for tyrosinase was added to the CPG-immobilized enzyme that has lost three fourths of its activity and shaken well. After keeping for about an hour, it was washed several times with the phosphate buffer and the activity was checked using tyrosine. The change in absorbance from 4 to 20 minutes was 0.046 indicating no appreciable increase in activity. The treatment with phenol was repeated and activity was checked again as before. This time the change in absorbance from 4 to 20 minutes was 0.037 corresponding to a loss of activity of 75.4% compared to the value when first used. This observation led to the conclusion that the inhibition of the enzyme by tyrosine is non-competitive and that it is not reversible.

Tyrosinase catalyzes the reaction of phenol and oxygen to give 1,2 dihydroxybenzene and then o-benzoquinone as has been illustrated in chapter I. If the reaction can be carried out in the presence of potassium hexacyanoferrate(II), the hexacyanoferrate(II) ions are oxidized to $Fe(CN)_6^{3-}$ ions with the concurrent reduction of o-benzoquinone to 1,2 dihydroxy benzene.



Consequently, the accumulation of hexacyanoferrate(III) ions in the system is directly related to the phenol concentration when the system reaches equilibrium and to the same concentration and the time in the kinetic region of the reaction. The accumulated hexacyanoferrate(III) ions can be spectrophotometrically detected at 420 nm [34]. Table XII shows the response of a sample of CPG-immobilized tyrosinase to 3.3×10^{-4} M phenol solution. As with the case of tyrosine, there is a progressive decline in the activity of the enzyme when 3.3×10^{-4} M phenol is used as a substrate. Three fourths of the activity has been lost on using the immobilized preparation seven times and almost four fifths of the activity has been lost when the same sample of the immobilized enzyme has been used repeatedly 12 times. Using another sample of CPG-immobilized tyrosinase, the activity was tested in the same way by using a more dilute solution (3.3×10^{-5} M) of phenol. Table XIII shows the results.

Even though the same sample of CPG-immobilized enzyme has been used repeatedly for 8 times, about 60% of the original activity of the enzyme was still retained. Phenol at a concentration of 3.3×10^{-5} M does not inhibit the enzyme as much as tyrosine or 3.3×10^{-4} M phenol. The phenol concentration in polluted water samples is usually much lower than this value. Consequently, the immobilized tyrosinase preparation can be used for the determination of phenols without fear of being completely inactivated.

TABLE XII

RESPONSE OF CPG-IMMOBILIZED TYROSINASE TO 3.3×10^{-4} M PHENOL

Use	Δ A 2-20 min	% of activity lost compared to 1st use
1	0.239	--
2	0.198	17.2
3	0.161	32.7
4	0.125	48.2
5	0.104	56.7
6	0.093	61.0
7	0.059	75.0
8	0.062	74.0
9	0.057	76.0
10	0.054	77.4
11	0.045	81.1
12	0.042	82.5

TABLE XIII

RESPONSE OF CPG-IMMOBILIZED TYROSINASE TO 3.3×10^{-5} M PHENOL

Use	ΔA 2-20 min	% of activity lost compared to 1st use
1	0.114	--
2	0.080	29.7
3	0.078	31.8
4	0.076	33.0
5	0.063	44.4
6	0.067	41.3
7	0.075	34.2
8	0.070	38.7

Four different samples of mushroom were tested for tyrosinase activity using 3.3×10^{-5} M phenol as substrates. Two of the samples were procured from local grocery stores. One of the other samples was picked by the author's son from the back yard of their apartment. This one was a large variety. A smaller variety was also obtained locally. The mushroom was washed repeatedly with water and then soaked in the phosphate buffer to extract out any coloring matter. The results obtained are given in Table XIV. The change in absorbance values between 2-20 minutes have been normalized for 1.0 g weight of the sample.

TABLE XIV

COMPARISON OF THE TYROSINASE ACTIVITY OF VARIOUS SAMPLES OF MUSHROOMS WITH CPG-IMMOBILIZED TYROSINASE

Sample	Source	ΔA^* (2-20 min)
1.	Local (large)	0.799
2.	Local (small)	0.205
3.	Miami, Oklahoma	0.387
4.	DelVal, Pennsylvania	0.693
5.	CPG-Immobilized Tyrosinase	0.508

*ΔA values normalized for 1.0 g of sample

The tyrosinase activity of the mushroom varied from sample to sample. The extract from the mushroom obtained by soaking the samples in phosphate buffer tested negative for tyrosinase activity.

CHAPTER V

DETERMINATION OF PHENOL IN CONTINUOUS-FLOW SYSTEMS

Introduction

The enzyme tyrosinase immobilized on CPG via diazo coupling has been found to retain over 60% of its initial activity after being used repeatedly with 3.3×10^{-5} M phenol solution. Phenol concentrations in water samples are considerably lower and it was expected that the inhibition of the enzyme due to higher concentration of the substrate would be minimal. The following sections describe the construction of open tubular reactors containing immobilized tyrosinase and the results obtained when the OTRs were used for the determination of phenol in continuous-flow systems.

Silylation of CPG Using Toluene and Construction of Open Tubular Enzyme Reactors

Materials and Reagents

Most of the materials and reagents used have already been described in the previous chapter. The Teflon tubing (i.d. 1.02 mm) used to circulate the sample and reagents

through the flow system and the Tygon tubing (formulation S-54-HL, i.d. 1.30 mm, o.d. 2.30 mm) used to construct the enzyme reactors were procured from Cole-Parmer (Chicago, IL). "StandARd" Phenol solution (1 ml = 1 mg phenol) was obtained from Mallinckrodt (Paris, NY).

Apparatus

Tygon tubing was heated in a Lab-Line Imperial IV laboratory oven (Lab-Line Instruments, Meltrose Park, IL). A Beckman DB-GT spectrophotometer was used as detector with a model E-178-Q-10 (Markson Science, Inc., Del Mar, CA) flow cell of 10 mm path length and 80 ul chamber volume. A Hewlett Packard Model 17503A strip chart recorder was used as readout unit. Continuous-flow was maintained with the aid of an Ismatic mv-ge peristaltic pump. A Rheodyne Model 5041 4-way Teflon rotary valve (Rheodyne, Inc., Cotati, CA) was used for sample injection. Temperature studies were performed with the aid of a Lauda K-2/R constant-temperature bath (Brinkman Instruments, Westbury, NY) with a glass circulating water bath.

Procedure for Thermal Embedding of AA-CPG

A sample of AA-CPG was prepared using the procedure described in Chapter IV. Tygon tubing of about 100 cm length was filled with this AA-CPG. The tubing was tightly

packed using an electric vibrator. It was then wound around a glass tube and secured with metal wires so as to retain the coiled shape. This assembly was heated in the oven at 175 ^{o}C for 5 minutes. After allowing to cool, the coil was unwound and the unattached particles of glass were shaken loose by aspirating at one end and by using the electric vibrator. A stream of nitrogen gas was passed to remove all loose particles. The tubing was then wound onto the glass tube and secured using Scotch© tape. The reactor thus prepared had a coil diameter of 1.3 cm.

Procedure for diazotization

The coiled tubing with the AA-CPG embedded to the inner walls was then kept in a bath of ice and connected to a peristaltic pump. 2.0 g of $NaNO_2$ was dissolved in 100 ml of 2.0 M HCl and the beaker containing this solution was also kept in a bath of ice. This mixture was then pumped for about 45 minutes through the tygon tubing to which the AA-CPG had been embedded. Finally, the reactor was washed with cold 0.10 M KH_2PO_4 buffer of pH 6.5, by pumping the buffer through the tubing.

Procedure for Immobilization

About 20 ml of a tyrosinase solution containing 50 units of the enzyme/ml prepared in 0.10 M potassium phosphate buffer, was placed in a 25 ml volumetric flask.

This enzyme solution was circulated through the Tygon tubing containing the diazotized AA-CPG in a closed loop system. The volumetric flask as well as the coiled tubing were maintained at a temperature of approximately 4 ^{o}C. This reaction was allowed to continue for about 45 hours at the end of which the buffer was pumped through the system for about 30 minutes to flush out any free enzyme. Two reactors of 50 cm length each were cut from the one that has been prepared and were used in further studies.

Spectrophotometric Determination of Phenol

The oxidation of phenol by tyrosinase takes place in the presence of molecular oxygen. Therefore, prior to any determination of phenol, the sample solution was aerated by passing air through the solution for about 10 minutes to saturate it. A mixture of the phenol solution and 1.0 x 10^{-2} M $K_4Fe(CN)_6$ both prepared in the buffer constituted the sample. Initial studies were carried out using 50 µg ml^{-1} phenol solution. The reactor was attached to the sample loop position of the injection valve and 0.10 M phosphate buffer of pH 6.5 was used as the carrier. Figure 2 shows the injection valve illustrating the position of the reactor. A flow rate of 3.6 ml/min was used because it gave narrow and reproducible flow injection peaks. The reactor was first flushed by injecting about 2

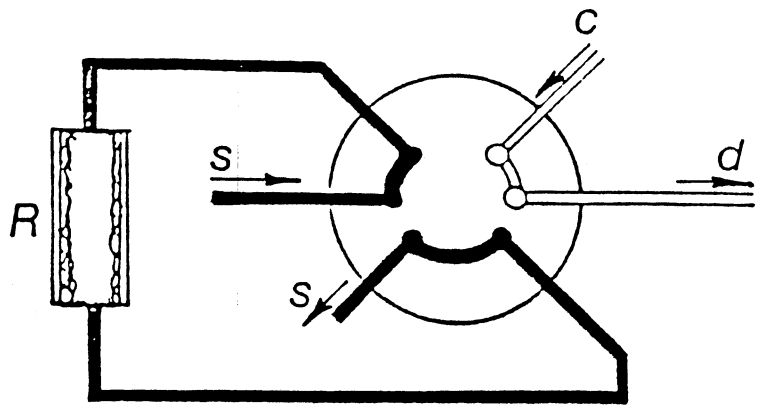


Figure 2. Injection Valve Showing Position of the Enzyme Reactor in the Sample Loop

R: reactor; ss: sample loading path
cd: carrier path to detector

ml of the sample. Since it was learned from earlier studies that the enzyme has a lag period of about 2-3 minutes, the sample was allowed to remain within the reactor for at least two minutes and then flushed out.

The sample solution was drawn into a disposable syringe and then injected so as to fill the reactor loop. The phenol sample was allowed to remain in the loop and react with the enzyme for a period of one minute. A stop watch was used to time "exactly" one minute, after which the sample was injected and the flow injection peak was recorded. As many as 6-8 injections were made using the same sample. Peak heights were measured and the average peak height determined. After use, the reactors were washed by flushing with the buffer and stored in the freezer compartment of the refrigerator.

Results and Discussion

The flow injection peaks obtained were very short showing very little response towards the phenol. Even when the phenol concentration was increased to as much as 300 μg ml^{-1}, no increase in the peak height was noticed. Either no enzyme or only very little had been attached to the CPG. The small peaks obtained could have been due to the small amount of hexacyanoferrate(III) formed by the air oxidation of hexacyanoferrate(II). Since tyrosinase has been successfully immobilized by this procedure, one

possible explanation could be that the AA-CPG on heating to 175 ^{o}C loses the aminosilane groups preventing the formation of the diazo salt.

Silylation of CPG Using Ethanol and Construction of Open Tubular Enzyme Reactors

Silylation of CPG has also been carried out using ethanol [150,151]. Therefore an attempt was made to try this alternate route since silylation in ethanol can be carried out without heating.

Procedure for Silylation Using Ethanol

CPG was embedded to the inside walls of the tygon tubing in essentially the same manner as has been described earlier for the thermal embedding of AA-CPG. A scanning electron micrograph of a Tygon reactor with the CPG particles embedded on the inside walls is given in Figure 3. A 10 % solution of (aminophenyl) trimethoxy silane in ethanol was prepared by dissolving 3 ml of the silane in 27 ml of anhydrous ethanol. The coiled tygon tubing to which the CPG had been embedded was connected to a peristaltic pump and the silane solution was pumped through the tubing at a slow rate of less than 1 ml/minute for approximately 45 minutes. About 10 ml of anhydrous ethanol was then pumped through to wash away any unreacted aminosilane.

Figure 3. Scanning Electron Micrograph of a CPG-embedded Tygon Reactor

Heating temperature: 175 ^{o}C
Heating time: 5 min
Magnification: x68

Procedure for Diazotization and Immobilization

These two steps were carried out using the same procedure as has been described before. One modification that has been made in this case was that the ends of the tubes were closed to keep the enzyme inside and the tubing was stored in the refrigerator for about eight hours each during two nights when the reaction was still in progress. During the day, it was attached to a peristaltic pump and the enzyme solution was circulated in a closed loop system through the tubing. At the end of about 45 hours, 0.10 M phosphate buffer of pH 6.5 was pumped through the system for about 30 minutes to flush out any free enzyme.

Spectrophotometric Determination of Phenol

A 60 cm length reactor corresponding to a sample volume of 420 ul was used in these studies. The reactor was attached to the loop position of the injection valve. Two different schemes were used for the continuous-flow determination of phenol in conjunction with spectrophotometric detection: single carrier scheme, and merging zone scheme.

Single Carrier Scheme

The standard phenol solution was diluted with the buffer and air was bubbled through a known volume (usually 10.0 ml) of this solution for about 10 minutes. A 1.0 x 10^{-2} M $K_4Fe(CN)_6$ solution was also prepared in the same buffer. A stream of nitrogen gas was passed through this solution to deoxygenate it. The aerated phenol solution was mixed with an equal volume of this hexacyanoferrate(II) solution to give the appropriate concentration. This way the chances of the slow oxidation of $Fe(CN)_6^{4-}$ was to some extent minimized if not eliminated. This mixture constituted the sample. 0.10 M KH_2PO_4 buffer of appropriate pH was used as the carrier. Figure 4 is a schematic representaion of the single carrier scheme.

Merging Zone Scheme

In this scheme, the hexacyanoferrate(II) solution, which was mixed with the sample of phenol, was introduced as a separate line. The carrier was the same buffer. The two solutions were merged through a "Y" connector and mixed together using a single bead string reactor (SBSR). The SBSR used consisted of glass beads filled in a coiled glass capillary tube. Figure 5 shows the manifold used in the merging zone scheme.

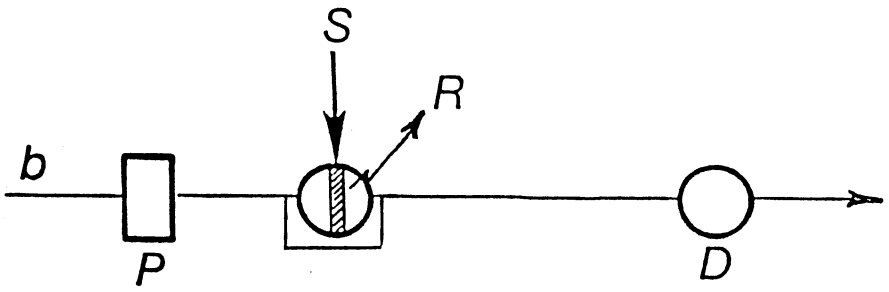


Figure 4. Single Carrier Scheme

b: carrier (buffer) stream
P: pump; S: sample; R: reactor
D: detector

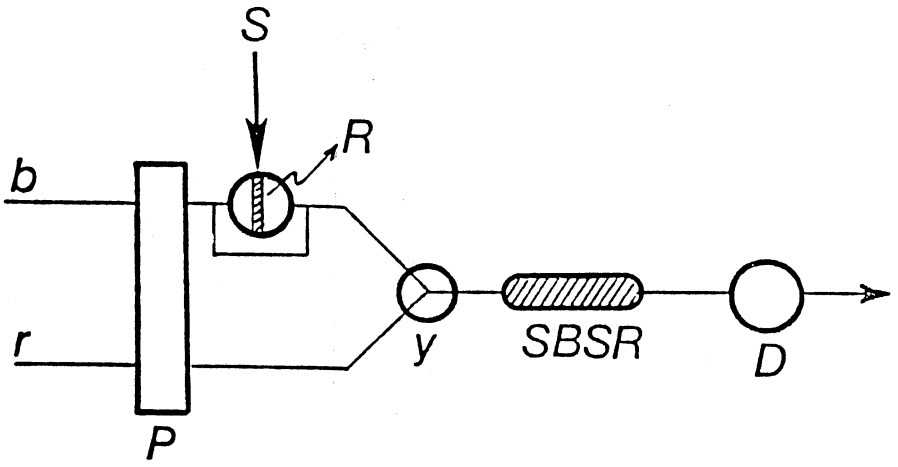


Figure 5. Merging Zone Scheme

b: buffer stream
r: hexacyanoferrate stream; P: pump
S: sample; R: reactor;
y: merging connector
SBSR: single bead string reactor (glass, 35 cm long, i.d. 1.5 mm, coil diam., 1.3 cm; packed with 1 mm diameter glass beads)
D: detector

Results and Discussion

Repeated Use of the Same Reactor

Two of the immobilized enzyme reactors were used once every day for several days under the same set of conditions. The initial response of the reactor has been normalized to give an arbitrary acivity value of 100 units and subsequent responses were converted to the corresponding values. The results obtained from one of the reactors is shown in Figure 6. There is a marked decrease in the activity with the first few uses. After the reactor has been used four times, the activity levels off and remains almost constant at about 85% of the original value. A similar result was obtained with the other reactor also.

Although the mechanism of how an immobilized enzyme retains most of the activity with time is not clearly known, it is widely believed that the covalent binding of the enzyme to the matrix reduces its denaturation by preventing the unfolding of the enzyme resulting in stability. It has been accepted that unfolding of the protein is the major cause of enzyme denaturation [152].

Single Carrier and Merging Zone Schemes

In the single carrier scheme, the $K_4Fe(CN)_6$ solution was added to the solution of phenol in the buffer that had been aerated and this sample was then used to fill

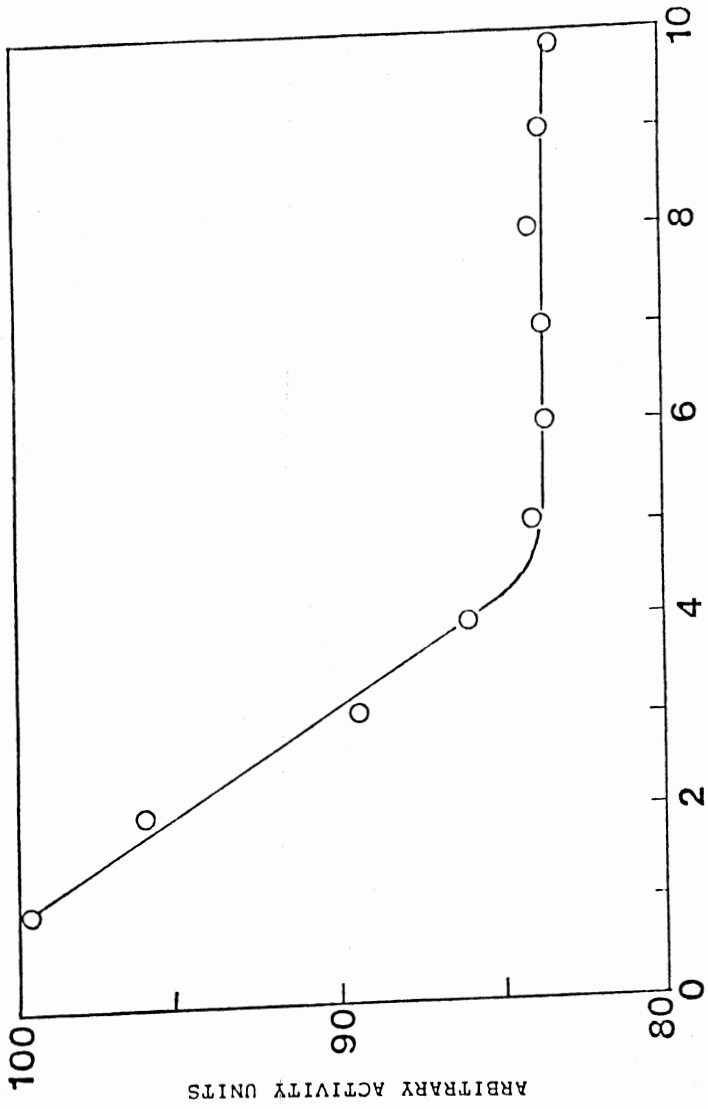


Figure 6. Repetitive Use of Immobilized Tyrosinase Reactor

the reactor. As $Fe(CN)_6^{4-}$ remains in contact with the phenol and oxygen, the uncatalyzed reaction (slow oxidation of hexacyanate(II) to hexacyanate(III)) may also be taking place. To avoid this problem, instead of using the buffer as a single carrier stream, another line (stream) was added through which $K_4Fe(CN)_6$ in buffer was pumped, and the two carrier solutions were later merged and mixed. When this merging zone scheme was tested, a reduction of about 35-40% in the average peak height was noticed compared to the peak height obtained when $Fe(CN)_6^{4-}$ was directly mixed with the sample. In spite of the decrease, there was very good reproducibility in the flow injection peaks.

pH Optimization Studies

The pH has a marked effect on the activity of enzymes and this in turn affects the rate of enzyme catalyzed reactions. The pH that corresponds to the maximum activity, called the optimum pH is of interest to the analytical chemist. A reactor was first conditioned by repeated use and then used in this study. 20 μg ml^{-1} solution of phenol that had been aerated for about 10 minutes was used throughout this experiment. 0.10 M KH_2PO_4 solution was prepared and its pH was adjusted to values between 6.0 to 8.5. Figure 7 is a plot of the pH vs average peak height. A broad pH optimum has been noticed between pH

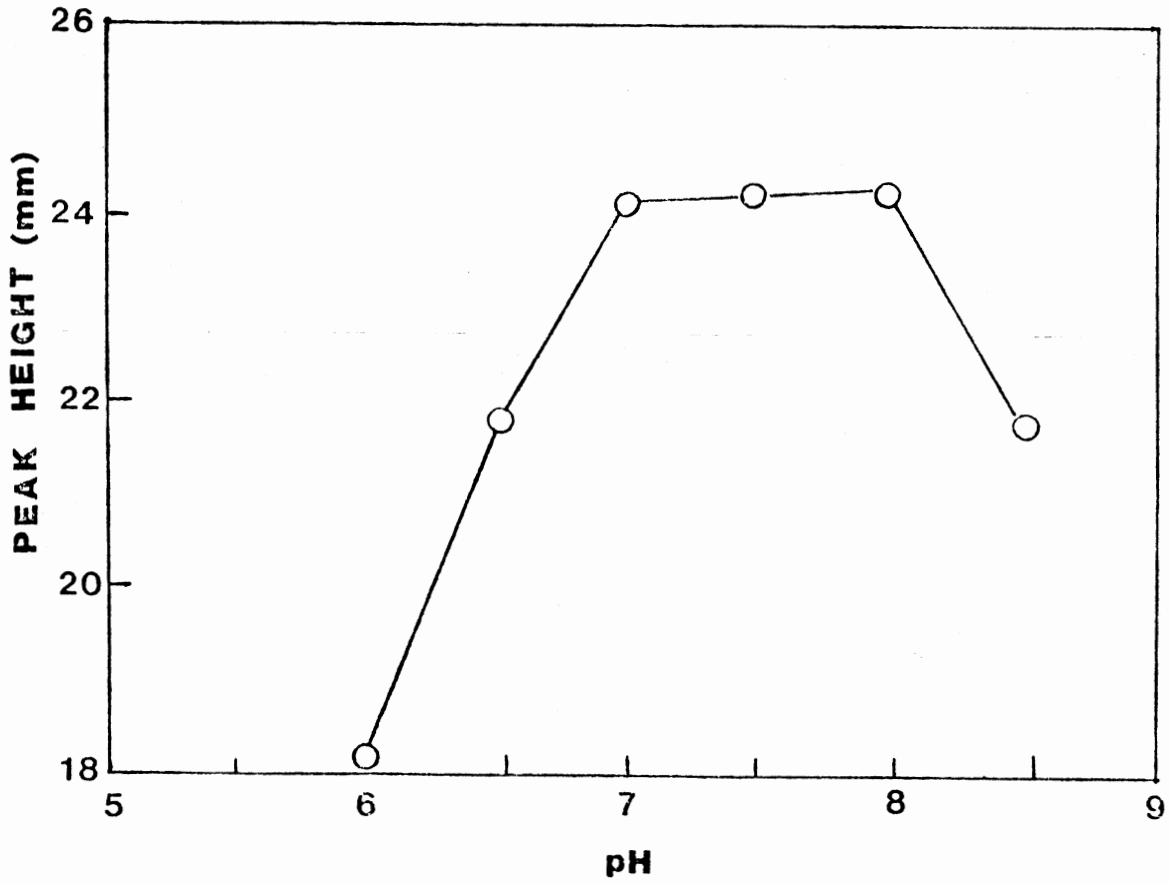


Figure 7. Effect of pH on the Peak Height

values of 7.0 and 8.0. An optimum pH of 7.5 has been used in further work.

Effect of Different Concentrations of Hexacyanoferrate on Peak Height

No change in peak height was observed by decreasing the hexacyanoferrate(II) concentration from 5.0×10^{-3} M to 2.0×10^{-3} M; but a decreasing trend was observed with mediator concentrations of 1.0×10^{-3} M or lower. A concentration of 2.0×10^{-3} M was adopted for further work to favor low blank readings and to reduce the effect of $Fe(CN)_6^{3-}$ produced by air oxidation.

Study of Different Buffers at pH 7.5

The responses obtained when three different buffers, each of pH 7.5, were used with the same reactor have been tabulated in Table XV. The merging zone scheme with two minutes contact time was employed with phenol of 20 µg ml^{-1} concentration. Triethanolamine hydrochloride buffer showed a slightly higher response than the potassium phosphate buffer whereas tris(hydroxy methyl)aminomethane buffer had a much lower response compared to the other two. Since there was not much difference in the responses between KH_2PO_4 buffer and triethanolamine hydrochloride buffer, for convenience potassium phosphate (monobasic) buffer of pH 7.5 was used in all further studies.

TABLE XV

RESPONSE OF IMMOBILIZED ENZYME REACTOR TO DIFFERENT BUFFER SYSTEMS

Buffer, pH =7.5	Avg. peak height* (mm)
0.10 M Potassium phosphate (monobasic) ..	17.0 ± 0.42
0.10 M Triethanolamine.HCl ..	17.3 ± 0.31
0.10 M Tris(hydroxy methyl)aminomethane ..	11.5 ± 0.29

* Average of 6-8 peak heights

Effect of Temperature

While this study was carried out, the injection valve was placed sideways with the reactor loop immersed in the water bath of the thermostat. Single carrier scheme and a contact time of 1 minute was used in this study with a phenol concentration of 20 $\mu g\ ml^{-1}$. Table XVI tabulates the responses obtained when a reactor was tested at different temperatures.

TABLE XVI

EFFECT OF TEMPERATURE ON IMMOBILIZED TYROSINASE REACTOR

Temperature	Avg. peak height* (mm)
20 °C	16.3 ± 0.46
25 °C	19.4 ± 0.35
30 °C	26.8 ± 0.54
35 °C	34.0 ± 0.82
40 °C	48.3 ± 0.77

* Average of 6-8 peaks

Figure 8 shows the flow injection profiles for this study. A three fold increase in reactor response was noticed when temperature was increased from 20 ^{o}C to 40^{o}C. However, the temperature was not increased beyond 40^{o}C so as not to destroy the enzyme. Increase in enzyme activity with temperature is in line with the behavior of immobilized enzymes.

Effect of Contact Time.

Using the single carrier stream and a phenol concentration of 50 ug ml^{-1} a set of two injections each were made by varying the reaction time from 1 minute to 6 minutes. The results from this study are given in Table XVII. A profile of the flow injection peaks is shown in Figure 9. The response of the reactor increased when the time the sample was allowed to remain and react with the enzyme was increased. The response almost doubled when the time was increased from one minute to two minutes.

Blank Readings and Limit of Detection

A solution of 2.0 x 10^{-3} M $Fe(CN)_6^{4-}$ in the phosphate buffer was injected through the system for blank measurements. The average peak height of ten blank readings was determined and the corresponding standard deviation was calculated. From this, the limit of detection was

TABLE XVII

EFFECT OF CONTACT TIME ON IMMOBILIZED TYROSINASE REACTOR

Time (minutes)	Peak height (mm)	
	Injection 1	Injection 2
1:00	22.3	21.9
2:00	44.2	44.4
3:00	56.0	56.0
4:00	63.7	63.9
5:00	72.4	72.0
6:00	79.7	80.3

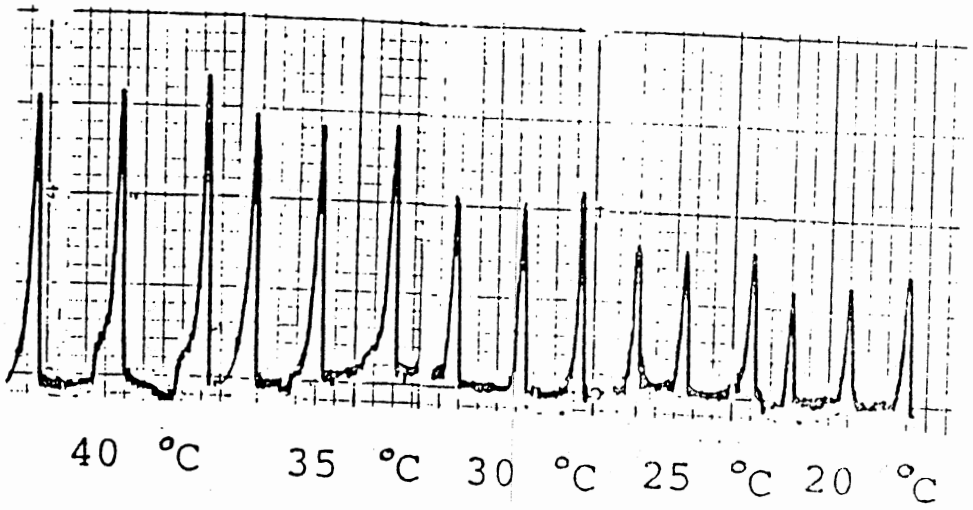


Figure 8. Signal response vs. Temperature

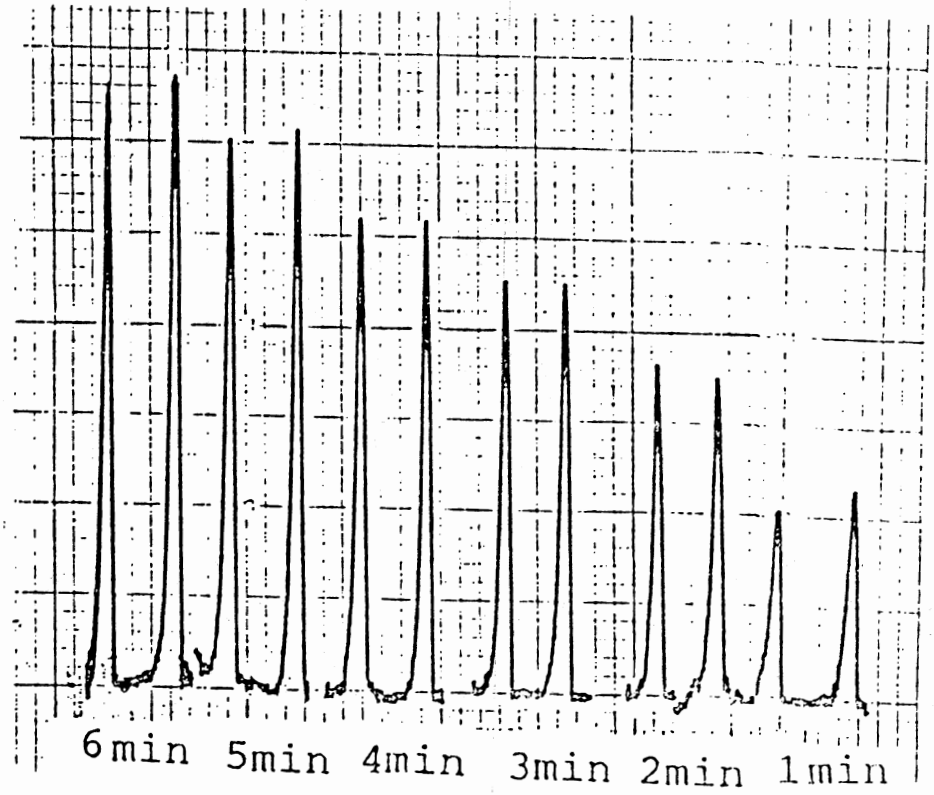


Figure 9. Signal Response vs. Contact Time

calculated as the concentration of phenol corresponding to the point 3 times the standard deviation from the average blank measurements.

Calibration Curves

Using the merging zone scheme and two minutes contact time, the enzyme reactor was used to prepare calibration curves with varying concentrations of phenol. Similar calibration curves were prepared using the single carrier scheme; but the sample was allowed to remain within the reactor for only one minute. Though there was an increase in reactor response with increase in temperature, the data for the calibration curves were collected at the laboratory temperature (ca. 22 ^{o}C). Calibration curves plotted according to the data are shown in Figure 10.

Both curves are linear up to a phenol concentration of about 40 µg ml^{-1} with correlation coefficients 0f 0.9966 and 0.9996. But the calibration curve for the single carrier stream showed a higher sensitivity and a lower limit of detection even though the sample was allowed to remain in the reactor for only one minute compared to two minutes in the other. The limits of detection were 6 µg ml^{-1} for the merging zone scheme and 3 µg ml^{-1} for the single carrier scheme. The intercept of the plot for the merging zone scheme was almost identical to the value obtained for the average peak heights when a blank $(Fe(CN)_6^{4-}$ in

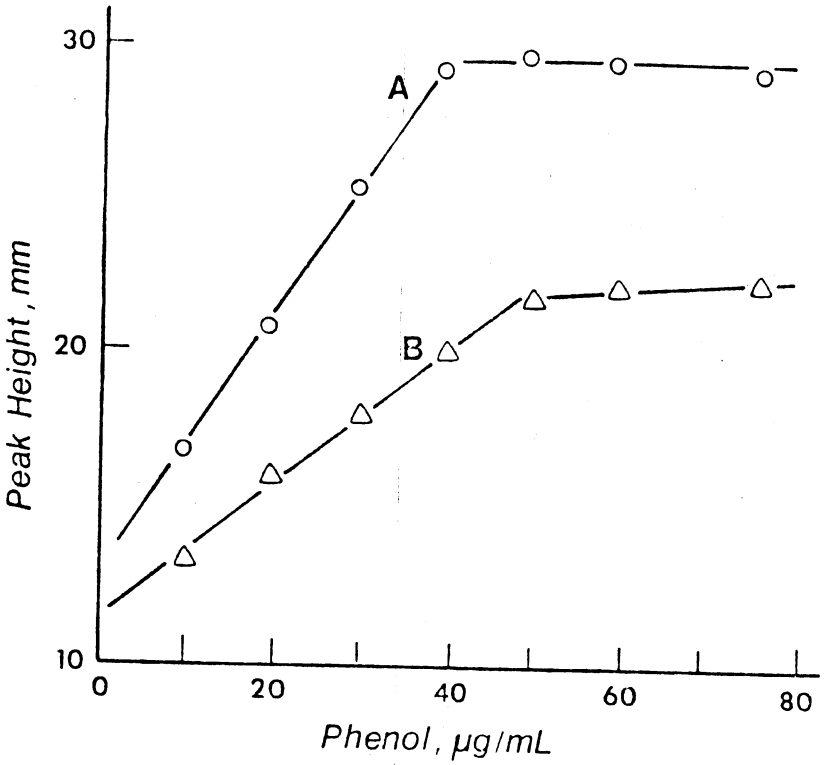


Figure 10. Calibration Curves (Spectrophotometric)

Curve A: Single carrier scheme with 1 min contact time

Curve B: Mercury zone scheme with 2 min contact time

buffer) was injected through the reactor. The slightly higher value for the intercept of the other plot may be due to the hexacyanoferrate(III) formed by the slow oxidation of hexacyanoferrate(II).

Amperometric Determination of Phenols

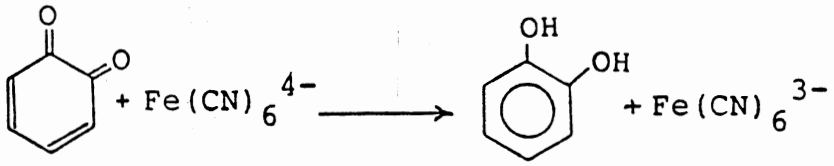
Electrochemical methods usually have higher sensitivity than spectrophotometric methods and can be applied to colored or turbid samples. Various electrochemical methods by which phenols have been determined were reviewed in Chapter III. Some of them [104, 106-108, 116, 119, 128] were amperometric detection methods used in conjunction with HPLC and the others [129-131] were direct methods.

The hexacyanoferrate reagent used as a redox mediator in this study not only provides the yellow color due to $Fe(CN)_6^{3-}$ for the spectrophotometric determination of phenol described above, but can also provide a Fe(II)/Fe(III) redox couple that can be used for electrochemical detection.

One of the advantages of immobilized reagents is the simplification of reagent handling; a fact that has been illustrated by the CPG-immobilized tyrosinase reactor. If the redox mediator can also be immobilized and used as part of an electrode, it would further simplify the whole analytical procedure for the determination of phenols. A convenient method for the immobilization of hexacyanoferrate

has been reported by Geno et al. [153]. When poly (4-vinylpyridine) or PVP is placed in a solution of hexacyanoferrate at a pH of 4.00, the pyridine moieties are protonated and $Fe(CN)_6^{4-}$ binds to the polymer.

This PVP-immobilized hexacyanoferrate can be conveniently incorporated into a carbon paste electrode by direct admixing. This modified carbon paste electrode can be used in the amperometric detection of the o-quinones resulting from the tyrosinase-catalyzed oxidation of phenols by measuring the current needed to convert the hexacyanoferrate(III) back to hexacyanoferrate(II). The reactions for o-benzoquinone given below illustrate this.



$$Fe(CN)_6^{3-} + \bar{e} \longrightarrow Fe(CN)_6^{4-}$$

Experimental Methods and Procedures

Apparatus.

Cyclic voltammetry studies were conducted with a BAS-100 Electrochemical Analyzer. A Model LC-4B unit was used as the amperometric detector and the output was recorded on a BAS X-Y recorder. All these instruments, and the reference electrodes (3 M Ag/AgCl) used in this study

were supplied by Bioanalytical Systems (West Lafayette, IN). The thin-layer cell, and the cell housing the reference electrode were fabricated from plexiglass at the Oklahoma State University machine shop.

Reagents and Materials.

Graphite powder of formulation UCP-1-M was supplied by Ultra Carbon (Bay City, MI), light mineral oil of viscosity 80-90 SUS at 100 ^{o}F was obtained from Sargent-Welch (Skokie, IL) and the poly (4-vinylpyridine) was procured from Reily Tar and Chemical Corp., (Indianapolis, IN). The potassium chloride was from J. T. Baker Chemical Co., (Philipsburg, NJ). The various phenols tested in this study were obtained from different sources. Aldrich Chemical Co. supplied 3-amino-p-cresol (97%), 3-ethylphenol (99%), 2-ethylphenol(99%), 2,3-diaminophenol (97%), m-cresol (99%), p-cresol (99%+), 4-ethylphenol (99%), and 3,5-dimethylphenol (99%). 2,4-dichlorophenol, and 2-chlorophenol were obtained from Sigma Chemical Co., (St. Louis, MO). The Chemical Division of Eastman-Kodak supplied 2,4-dimethylphenol, 2,4,6-trichlorophenol, and pentachlorophenol. 2,4-Dinitrophenol was procured from Fisher Scientific Co (Chicago, IL) and o-cresol was obtained from Matheson Coleman and Bell. All these phenols were used without further purification. Other materials and reagents used have been described elsewhere.

Immobilization of Hexacyanoferrate to Poly (4-vinylpyridine).

About 2.0 g of PVP was placed in about 20 ml 0.10 M potassium hexacyanoferrate(II) solution at pH 4.00 and contact aided by placing the beaker in an ultrasonic bath for a few minutes. The product was filtered and air dried.

Preparation of Modified Carbon Paste Electrode.

Carbon pastes were prepared by mixing 60% by weight of graphite powder with 40% by weight of light mineral oil which acts as a binder. The PVP-immobilized hexacyanoferrate was used to modify the carbon paste by direct admixing and the mixture was blended thoroughly in an agate mortar. Although 4% (w/w) and 8% (w/w) of PVP-immobilized hexacyanoferrate modifier in the carbon paste gave better sensitivity resulting in higher peaks, a 2% composition was preferred because the back ground current was then considerably lower. The modified carbon paste was then used to pack the cavity of a thin-layer cell and the surface was smoothed by pressing on a clean computer card. The thin-layer cell consists of two blocks each 2 cm x 2 cm made of Plexiglass^{R} with a thin plastic spacer which is approximately 0.10 mm in thickness between them. The cavity at the center of the lower block is only about 2 mm in

diameter. The upper block has an inlet and an outlet for the solution to flow in and out. A schematic diagram which shows the different parts of a thin-layer cell is given in Figure 11. A large extent of electrolysis is possible in thin-layer cells since they hold only a smaller volume. Thus the amperometric response from such a cell comes close to coulometric response resulting in higher sensitivity.

Instrumental Setup.

The lay out of the instrumental setup used for the continuous-flow amperometric determination of phenols is given in Figure 12. The carrier solution was taken in the reservoir and gravity was used for the flow. The injection valve was the same as the one used for photometric detection with the reactor attached to the sample loop position. The thin-layer cell with the modified carbon paste is the working electrode and a 3 M Ag/AgCl electrode served as the reference electrode. The metallic side arm of the cell housing the reference electrode is the auxiliary electrode. The potentiostat of the amperometric detector can be set at any desired potential. When an injection of the sample is made, it flows over the working electrode containing the redox mediator. An increase in current is noticed, which finally decays resulting in a flow injection peak, which is recorded on the strip chart recorder.

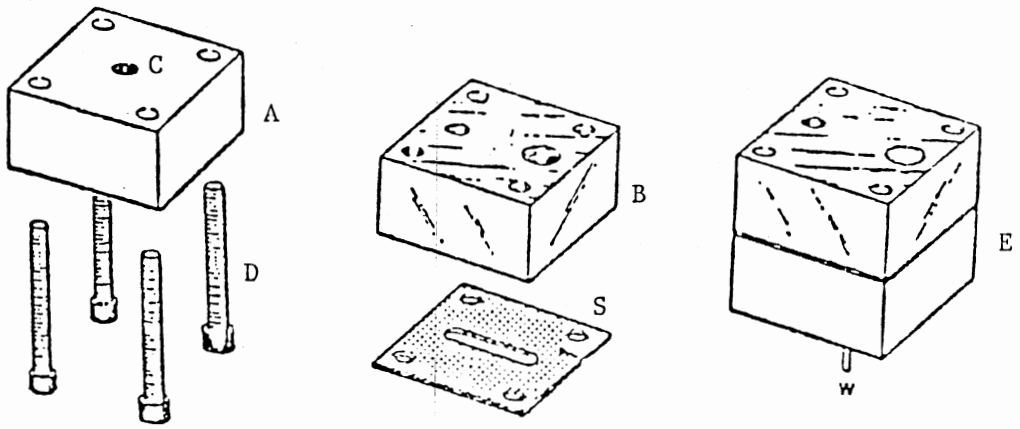


Figure 11. Schematic Diagram of a Thin-layer Cell and its components

A: Lower block; B: Upper block
C: Cavity; D: screw
E: Thin-layer cell (assembled)
S: Spacer

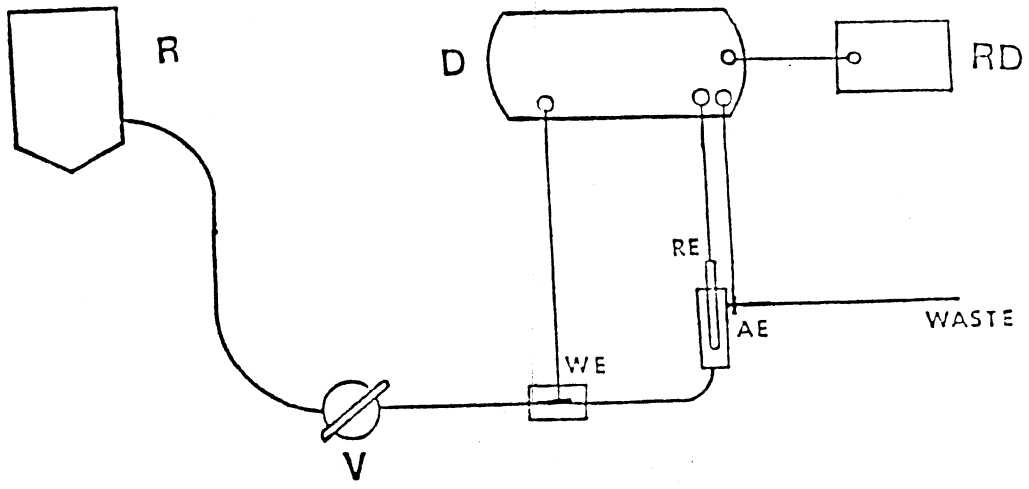


Figure 12. Instrumental Setup for Continuous-flow Amperometric Determination of Phenols

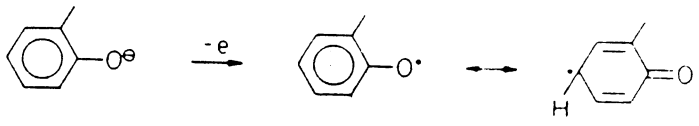
R: Reservoir; V: Injection valve
WE: Working electrode
D: Amperometric detector
RE: Reference electrode
AE: Auxiliary electrode
RD: Readout device

Results and Discussion

Electrochemistry of Phenol on Carbon Paste Electrode.

Phenol can be electrochemically oxidized on a carbon paste electrode at a relatively high electrode potential of +0.80 to +0.95 V vs 3 M Ag/AgCl. The signal obtained under a flow system by repetitive injections of 0.20 µg ml^{-1} solution of phenol on a carbon paste electrode at +0.90 V vs 3 M Ag/AgCl is shown in Figure 13. There is a rapid deterioration of the signals with time as evidenced by the decrease in peak height. This decrease is more pronounced when the phenol concentration is increased. Because of the fouling of the electrode surface by the deposition of the polymeric film produced by phenolate ions, carbon paste electrodes cannot be conveniently used for the detection of phenols. Mengoli [154] has suggested the following mechanism for the polymerization that takes place when phenols are electrochemically oxidized at glassy carbon or other carbon electrodes:

(a) initiaion:



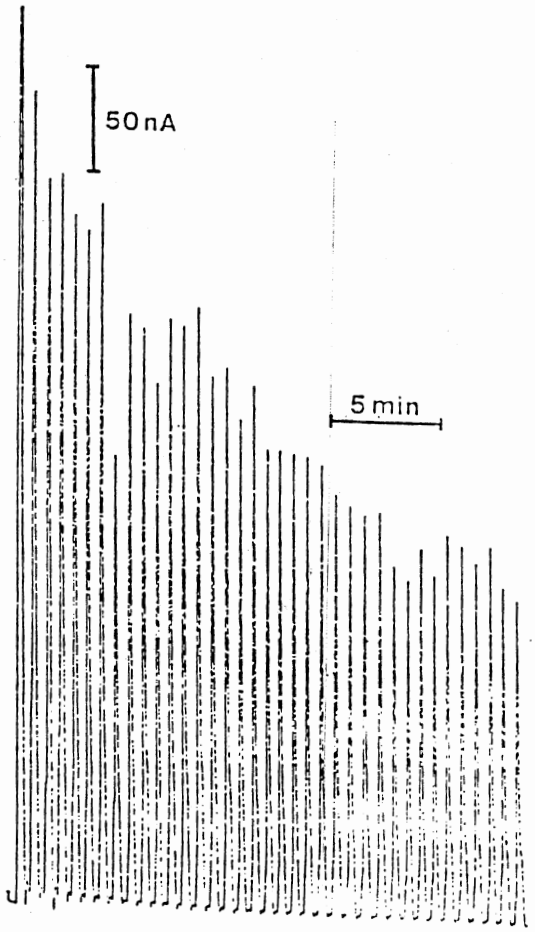
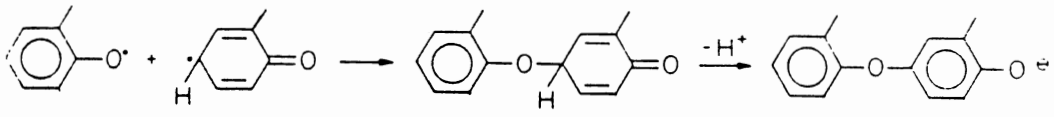


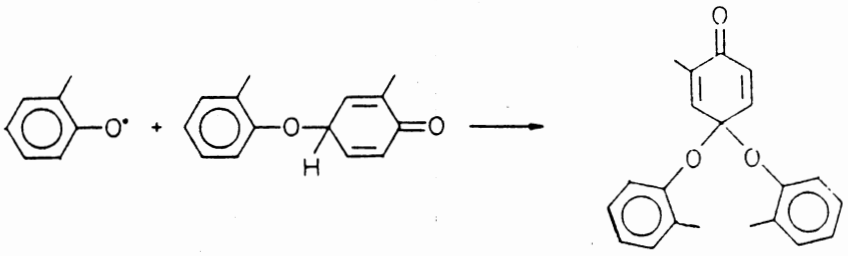
Figure 13. Signal Response of Phenol at a Carbon Paste Electrode

Phenol concentration: 0.20 μg ml^{-1}
Applied potential: + 0.90 V vs. Ag/AgCl
Flow rate: 1 ml min^{-1}

(b) propagation:



(c) termination



Cyclic Voltammetry Studies on PVP-Immobilized Hexacyanoferrate Redox Mediator.

Figure 14 is a cyclic voltammogram obtained wth a carbon paste electrode modified using 2% by weight of PVP-immobilized hexacyanoferrate. The oxidation potential of the Fe(II)/Fe(III) redox couple is close to typical values in solution ($E^{o'}$ value of +0.24 V vs 3 M Ag/AgCl) and is low enough to reduce damage from many redox interferences. The cyclic voltammetric behavior shows well developed waves with anodic peak-to-cathodic peak ratio of 1.03, which is typical for a reversible redox couple.

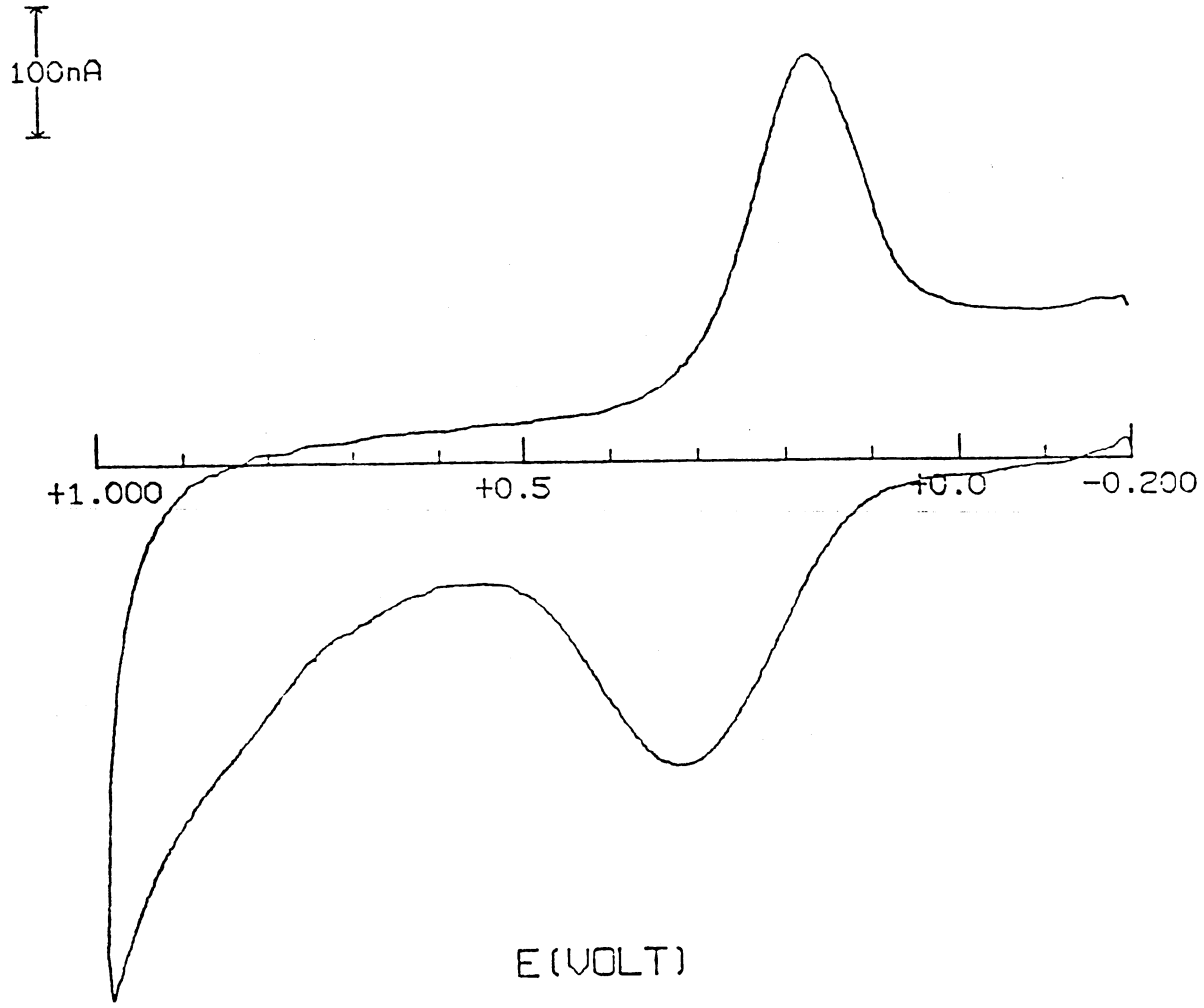


Figure 14. Cyclic Voltammogram of Carbon Paste Electrode Modified with PVP-immobilized hexacyanoferrate

Amount of modifier: 2% (w/w)
Supporting electrolyte: 0.10 M KCl; Scan rate: 50 mv s^{-1}

However, the ΔE_p value of 150 mV, is much higher than the normal value of 59 mV for a one electron process such as this; which indicates slow electron transfer. The immobilized redox mediator can thus be used for the amperometric detection of the o-quinone product resulting from the tyrosinase-catalyzed oxidation of phenols without the problem of surface poisoning observed with ordinary carbon paste electrodes.

Optimization of Reaction Conditions

The buffer system (0.10 M KH_2PO_4) and the optimum pH of 7.5 used in the spectrophotometric determination were not changed. The carrier used was the supporting electrolyte, 0.10 M KCl. The sample of phenol was prepared in a mixture of equal volumes of 0.10 M phosphate buffer and 0.10 M KCl.

Length of Reactor. The length of the reactor used is proportional to the sample volume. Whereas a 60 cm length reactor was used with spectrophotometric detection, a reactor of 20 cm length corresponding to a sample volume of 140 µl was sufficient for amperometric detection. Although peak heights obtained were smaller, a 10 cm length reactor could also be used. The sample volume is not that important in water analysis; but can be critical in some other instances when the amount of sample is limited. In such

cases, amperometric detection offers an advantage over spectrophotometric detection.

Applied Potential. A hydrodynamic voltammogram that shows the response of the immobilized tyrosinase reactor towards phenol at a carbon paste electrode modified with PVP-immobilized hexacyanoferrate when the applied potential was varied from +0.20 to -0.40 V (vs 3 M Ag/AgCl) is shown in Figure 15. A 20 cm length reactor was used with a phenol sample of concentration of 1 μg ml^{-1}. Curve 'a' is for a contact time of 20 s and curve 'b' is for a time of 30 s. A value of -0.200 V (vs 3 M Ag/AgCl) was chosen for the applied potential since the back ground current was lower at this potential than at -0.250 V or -.300 V. The response of the modified carbon paste towards phenol is at a much lower potential than that of the unmodified carbon paste which is beneficial to the decrease in interference from common redox species.

Flow Rate. The flow rate of the carrier stream affects the sensitivity as well as the sampling rate. Using phenol at a concentration of 0.50 μg ml^{-1} and a 20 cm length reactor, the effect of flow rate on the shape of the flow injection peaks were studied. The phenol solution was allowed to remain within the reactor for a period of 30 s before making the injection. The flow rate was varied by adjusting the height of the reservoir containing the carrier

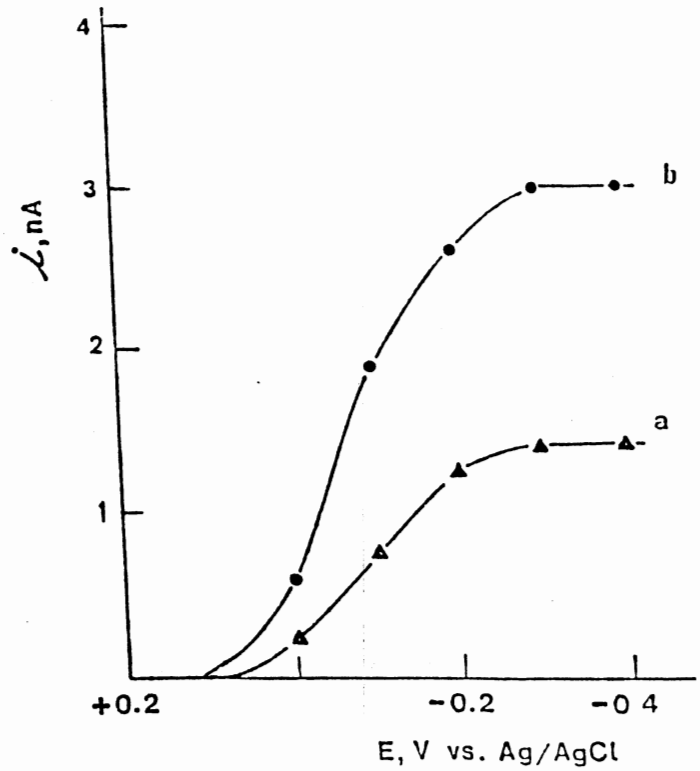


Figure 15. Hydrodynamic Voltammogram of Phenol at Modified Carbon Paste Electrode

Amount of modifier: 2% (w/w)
Phenol concentration: 1.0 µg ml^{-1}
Sample volume: 140 µl; Carrier: 0.10 M KCl
Curve a: Contact time of 20 s
Curve b: Contact time of 30 s

solution. The following table (Table XVIII) gives the results of this study.

TABLE XVIII

EFFECT OF FLOW RATE ON PEAK HEIGHT

Flow rate (ml/min)	Avg. Peak Height* (mm)
2.1	26.8 ± 0.64
2.8	28.1 ± 0.48
3.5	28.5 ± 0.63
4.2	26.9 ± 0.43
4.9	26.6 ± 0.67

*Average of 6-8 peaks.

A flow rate of 3.5 ml/min gave narrow peaks of optimum height and was adopted for further studies. This flow rate was comparable to the flow rate used with spectrophotometric detection. The high flow rate produced high sample through put and narrow flow injection peaks. Reduced sensitivity and additional quantity of reagents needed were, however, the trade off.

Contact Time. Using a phenol concentration of 0.50 μg ml^{-1} and a reactor length of 20 cm, the reaction time allowed for the sample to be within the reactor was varied from 10-30 s. There was sufficient response even when the contact time was as low as 10 s; but exactly timing 10 s intervals manually using a stop watch was not very convenient. Since perfect timing is highly important to attain good reproducibility, contact times of 20 s and 30 s were used when preparing calibration curves.

Calibration Curves.

Using the optimized reaction conditions and 20 s and 30 s as contact time, two sets of data were collected for phenol concentrations varying from 100 to 900 μg l^{-1}. The results were plotted and the calibration curves can be found in Figure 16. Curve A is for a contact time of 30 s and curve B is for a contact time of 20 s. Both curves show good linearity and offer low limits of detection (correlation coefficients: 0.9955 and 0.9954; limit of detection: 16 μg l^{-1} and 25 μg l^{-1}, respectively). The sensitivity was about 56% higher with 30 s contact time. There was excellent precision as evidenced by typical relative standard deviations of about 2% for 6-10 replicates.

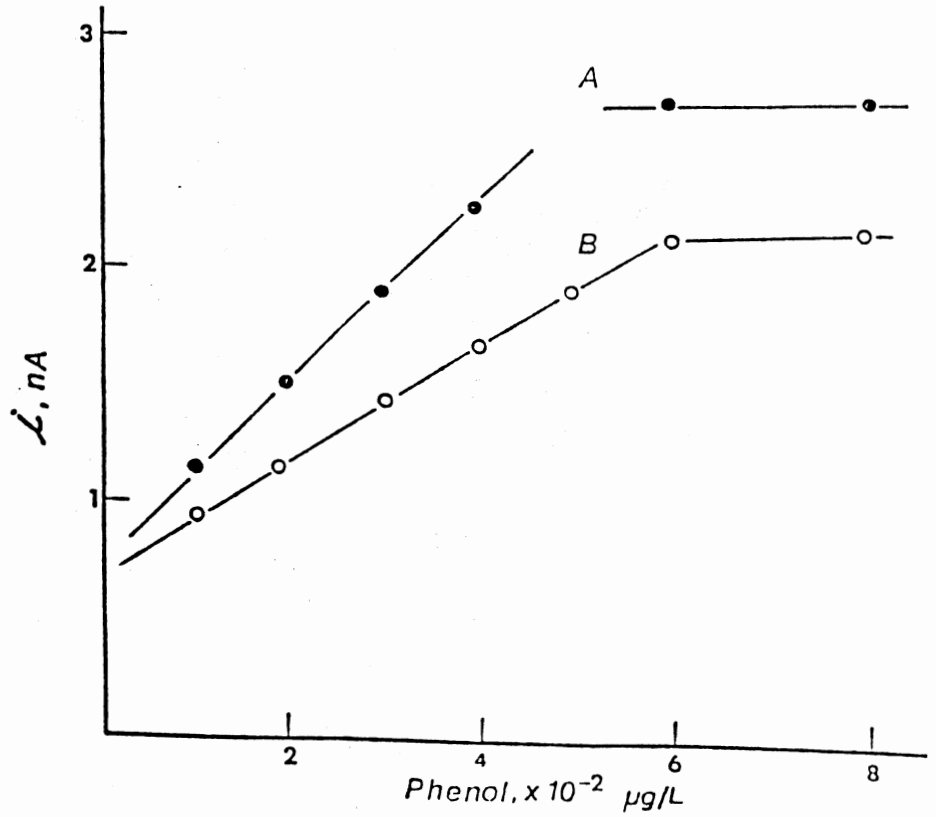


Figure 16. Calibtion Curves (Amperometric)

Curve A: Contact time of 30 s
Curve B: Contact time of 20 s

Analysis of EPA Quality Control Water Sample

A water sample provided by the U.S. EPA (Water Pollution Quality Control Sample WP 785) was diluted to a volume that provided a concentration that will be in the linear range of the calibration curve B, and the phenol content was determined under the same conditions. The results are given in the following table (Table IXX).

TABLE XIX

RESULTS OF ANALYSIS OF EPA QUALITY CONTROL WATER SAMPLE

Value in injected EPA sample, $\mu g\ l^{-1}$	Value found $\mu g\ l^{-1}$
180	171
225	214
360	351

Although the phenol concentration obtained was consistentlty slightly lower, the correlation between the

reported and found values is satisfactory. The lower results could probably be due to the decomposition of phenols during storage and sample preparation.

A comparison of the results obtained in the spectrophotometric and the amperometric detection methods is given in Table XX. Various aspects of the continuous-flow determination of phenol using immobilized tyrosinase have been enumerated in a recently published paper [155].

Even though the sample volume is not critical in water analysis, the spectrophotometric detection technique used three times as much sample as the amperometric detection method. Being more sensitive, the detection limits achieved were also much lower in the amperometric method even though contact times were reduced up to 20 s. The contact time also has a bearing on the number of samples that can be processed per hour. Using the amperometric technique, up to 80 samples per hour can be processed compared to 25 and 30 samples per hour in the spectrophotometric method.

Determination of Other Phenolic Compounds

So far only L-tyrosine and phenol have been studied as substrates with the immobilized tyrosinase. There are other phenolic compounds that pollute water supplies and to study the response of immobilized tyrosinase to such compounds, a number of other phenols have been tested using the continuous-flow amperometric detection method under

TABLE XX

COMPARISON BETWEEN THE SPECTROPHOTOMETRIC AND AMPEROMETRIC METHODS

	Spectrophotometric		Amperometric	
	Merging Zone	Single Carrier	A	B
Contact time	2 min	1 min	30 s	20 s
Sample volume (µl)	425	425	140	140
Flow rate (ml/min)	3.6	3.6	3.5	3.5
Corr. Coefficient	0.9966	0.9996	0.9955	0.9954
LOD (µg ml^{-1})	6.0	3.0	0.016	0.025
Sampling Rate	25/h	35/h	60/h	80/h

identical conditions. The results obtained from such a study have been tabulated in Table XXI. All the phenolic compounds except phenol were insoluble or only sparingly soluble in water. They were first dissolved in the minimum needed quantity of ethanol and then diluted with water to make a stock solution. The sample solution of each of the phenolic compounds was prepared in a 1:1 mixture of 0.10 M KCl and 0.10 M KH_2PO_4 buffer of pH 7.50 to give a concentration close to 0.50 μg ml^{-1}. The carrier solution used in this study was a mixture of 0.10 M KCl and 0.10 M buffer to avoid having a blank reading. The other experimental parameters were: sample volume, 140 ul; contact time: 30 s; applied potential. -0.200 V vs. 3 M Ag/AgCl; and flow rate: 3.5 ml/min.

Compounds with both the ortho positions, with respect to the phenolic -OH group, occupied by other groups or atoms (2,4,6-trichlorophenol, and pentachlorophenol) did not give any response. Compounds such as 2-methylphenol, 2-ethylphenol, and 2-chlorophenol that have just one ortho position free gave only a low response. The most pronounced effect was on the para substituted phenols: 4-methylphenol and 4-ethylphenol.

The selectivity of the enzyme tyrosinase towards phenolic compounds coupled with the signal enhancement due to the hexacyanoferrate mediator in the thin-layer cell configuration provides a sensitive method for the direct amperometric determination of phenols in water samples. The

TABLE XXI

RESPONSE OF IMMOBILIZED TYROSINASE
TO DIFFERENT PHENOLIC COMPOUNDS

Phenolic Compound	Avg. Peak Current* (nA)
Phenol	2.4 ± 0.060
2-methylphenol	1.3 ± 0.038
3-methylphenol	4.3 ± 0.087
4-methylphenol	23.8 ± 0.380
2-ethylphenol	1.2 ± 0.029
3-ethylphenol	3.4 ± 0.098
4-ethylphenol	22.4 ± 0.390
3,5-dimethylphenol	1.5 ± 0.028
2-chlorophenol	1.2 ± 0.027
3-aminophenol	1.4 ± 0.035
2,3-diaminophenol	2.6 ± 0.047
2,4-dimethylphenol	-
2,4-dinitrophenol	-
2,4-dichlorophenol	-
2,4,6-trichlorophenol	-
Pentachlorophenol	-

*Average of 6-8 measurements

Sample concentration: 50 µg ml^{-1}; Volume: 140 µl
Contact time: 30 s; Flow rate: 3.5 ml min^{-1}
Applied potential: -0.200 V vs. 3 M Ag/AgCl

possibility of incorporating this method into other continuous-flow systems such as liquid chromatography for the detection of phenolic compounds is worth exploring.

CHAPTER VI

CONCLUSIONS

The enzyme tyrosinase from mushroom has been immobilized on CPG via diazo coupling. Although silylation of CPG using toluene and subsequent immobilization posed no problems, attempts to immobilize the enzyme inside Tygon tubing after thermally embedding AA-CPG, were unsucessful. But the procedure using ethanol for silylation could be adapted as an alternate route and OTRs containing immobilized tyrosinase have been successfully constructed. With the enzyme reactor as the sample loop, continuous-flow sample processing could be used to determine $\mu g\ ml^{-1}$ and $\mu g\ l^{-1}$ concentrations of phenol using spectrophotometric and amperometric detection methods.

Immobilized tyrosinase, being rather slow in the rate of its reaction with phenols, could not produce sufficient signals when the enzyme reactor was connected on line. This problem was overcome by using the reactor as the sample loop and by manipulating the contact time to improve sensitivity and thus to lower the limit of detection. This strategy was advantageous especially with spectrophotometric detection whose sensitivity is inherently lower than that of electrochemical methods.

Hexacyanoferrate has been found to be a versatile reagent as a redox mediator both for spectrophotometric and amperometric detection methods. The amplification effect resulting from the accumulation of hexacyanoferrate(III) when the mediator was mixed with the phenol and introduced into the single carrier scheme resulted in a higher sensitivity for this strategy; but the resulting calibration curve had a higher intercept compared to the merging zone scheme.

Use of PVP-immobilized hexacyanoferrate redox mediator as part of a modified carbon past electrode offers a number of advantages over its use in solution. In addition to the convenience afforded in handling the reagent, the pseudo-catalytic cycle offered by the hexacyanoferrate provided higher sensitivity and helped to lower the limit of detection of the amperometric detection method.

By immobilization, the enzyme retained the activity over a period as long as one year. Each enzyme reactor could be used over and over again which helped to spread the total cost over a large number of determinations. The flow injection manifold is simple to assemble even in a small laboratory and the instrumental set up used in the amperometric detection method is not only less expensive but also fast with competitive limits of detection.

One draw back of this tyrosinase based method is that the ortho position with respect to the -OH group of the phenol must be available for oxidation. Consequently

compounds such as 2,4,6-trichlorophenol, and pentachlorophenol can not be determined.

Continuous-flow sample processing systems is one of the analytical techniques that can be easily automated. With an automatic sample injection system, the problem of perfectly timing injections at 10 seconds or 15 seconds interval will not pose any problem. This in turn can increase the number of samples that can be analyzed per hour to 100 or more, resulting in still lower cost per determination.

In this study, only controlled-pore glass was used as the support matrix to attach the enzyme. Immobilization of tyrosinase on CPG of different pore sizes or even other inert supports could be attempted. Perhaps more enzyme loading could be achieved on another type of support; in which case, the reactor could be connected on line rather than as the sample loop. If an immobilized form of tyrosinase and the immobilized hexacyanoferrate can be directly admixed with the carbon paste, it would be possible to eliminate the reactor altogether. But CPG is not suitable for direct admixing with the carbon paste since it does not form a smooth paste. Polymeric materials that might form a gel could probably be used to immobilize the enzyme. Such avenues are worth exploring in the future.

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VITA

Kuruvilla Zachariah

Candidate for the Degree of

Doctor of Philosophy

Thesis: ANALYTICAL APPLICATIONS OF IMMOBILIZED TYROSINASE FOR PHENOL DETERMINATION

Major Field: Chemistry

Biographical:

Personal Data: Born in Kerala, India, January 17, 1948, son of Soosannamma and C. Kuruvilla.

Education: Received Bachelor of Science degree in Chemistry from University of Kerala, India, in 1966; Master of Science degree in Chemistry from Ravishankar University, Raipur, India, in 1970; completed the requirements for the Doctor of Philosophy degree at Oklahoma State University, Stillwater, Oklahoma in December, 1989.

Professional Experience: Lecturer in Chemistry, St. Stephen's College, Uzhavoor, Kerala, India, 1970-1972; Senior Secondary School Science Teacher, Ethiopia, 1972-1976; Lecturer, Addis Ababa University, Ethiopia, 1976-1977; Senior Secondary School Science Teacher, Kaduna State, Nigeria, 1977-1984; Graduate Teaching/Research Assistant, Department of Chemistry, Oklahoma State University, January, 1985-August, 1989; Assistant Professor of Chemistry, St. Mary of the Plains College, Dodge City, Kansas, since August, 1989.