United States
Environmental Protection
Agency

Office of Research
and Development
Washington DC 20460

EPA/600/R-96/137
November 1996

EPA

Detection of Phenols using Liquid Chromatographic System with an Enzyme-based Biosensor

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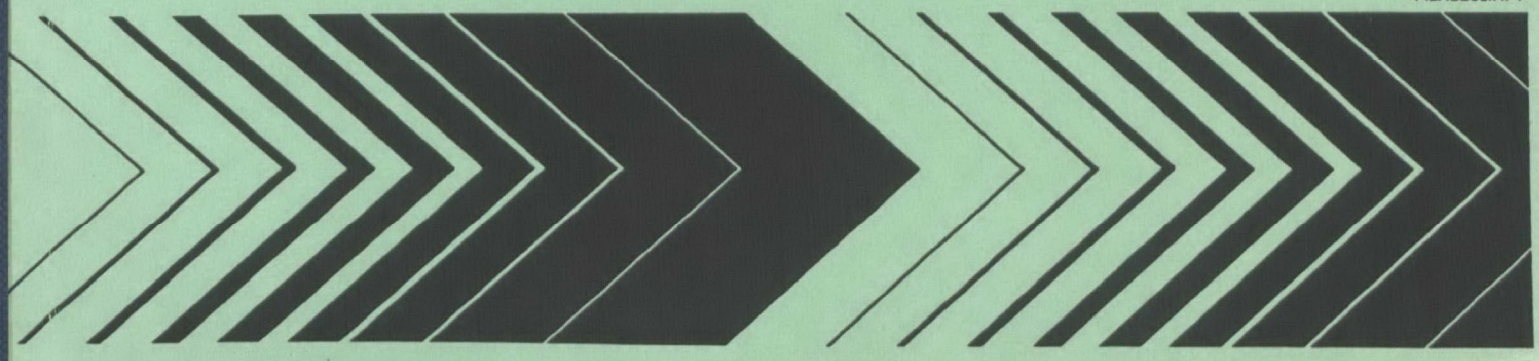

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Detection of Phenols using Liquid Chromatographic System with an Enzyme-based Biosensor

by

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Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development (ORD), has funded and managed the research described here. It has been subjected to the Agency's peer review and has been approved as an EPA publication.

Foreword

One of the approaches for reducing uncertainties in the assessment of human exposure is to better characterize the hazardous wastes which contaminate our environment. A significant limitation to this approach, however, is that sampling and laboratory analysis of contaminated air, water, and soil, is slow and expensive, thus limiting the number of samples which can be analyzed within time and budget constraints. In cases where a limited number of target analytes can be identified, faster and more cost-effective field screening and monitoring methods can potentially increase the amount of information available concerning the location and concentration of pollutants which may impact human health and the environment.

Due primarily to their operational format versatility, biosensors composed of biological recognition elements interfaced with optical, electrochemical, or acoustic signal transducers, show the potential to provide solutions for the previously mentioned limitations. In particular, the enzyme electrodes may fill in some of the gaps currently found in the field analytical technologies matrix. Some of the potential application areas for which these biosensors might be developed include: *in situ* monitoring for on-line process control for pollution prevention and remediation scenarios; sentinel capabilities for detection of episodic releases or continuous well monitoring in hazardous waste site post-closure monitoring scenarios; and detectors for field chromatographic systems which use detergent micelles or organic-based solvent systems.

The following research efforts, supported through the National Exposure Research Laboratory (NERL), Characterization Research Division (CRD-LV), Analytical Sciences Branch (ASB), are intended to develop and demonstrate electrochemical biosensors for detection of environmental pollutants such as phenolics. This work is part of a broader effort in which biosensors are being developed to function in potential applications which require portable, continuous, and *in situ* monitoring of aqueous and organic-based media.

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Chapter 1
Goal

The objective of this project is to characterize, optimize and test a dual-electrode detector for monitoring phenolic pollutants in liquid chromatographic effluents. Particular attention is given to the simultaneous use of a bioelectrode and conventional electrodes for the detecting of phenolics.

Chapter 2
Background

Because of the inherent toxicity of phenolic compounds, there is considerable interest for their determination in environmental matrices. Such compounds are reported at over 80 Superfund sites, and are listed on the Agency for Toxic Substances and Disease Registry's (ATSDR's) Priority Hazardous Compound List. Electrochemical sensors and detectors are extremely attractive for monitoring phenolic compounds. The phenol moiety can be oxidized at moderate potentials (ca. +1.0V vs. Ag/AgCl reference) hence opening the way for direct anodic detection of phenols (1). In addition, the enzymatic activity of tyrosinase (polyphenol oxidase, EC 1.14.18.1) can be coupled with amperometric transduction to yield effective biocatalytic sensors for phenols. Such a biosensing operation relies on the enzymatic conversion of phenols to quinones and subsequent low-potential reductive detection of the quinone product (2, 3).

This project employs both the direct- and enzymatic amperometric schemes for monitoring phenolic compounds in chromatographic effluents. Reverse-phase liquid chromatography represents a very useful tool for the separation of phenols. Dual-electrode amperometric detection for HPLC can offer additional information towards the identification of eluting (and coeluting) compounds (3). Commonly, such a detection scheme relies on the use of identical working electrodes, operated at different potentials, in connection with the parallel thin-layer cell configuration. In this project, the same detector configuration was employed, but in connection with a tyrosinase electrode (held at -0.2V) and a plain carbon electrode (operated at +1.2V). Such a unique dual-electrode detection mode adds a new dimension of information and selectivity, and hence greatly enhances the high performance liquid chromatography (HPLC) detection of phenolic pollutants.

Chapter 3
Experimental

Reagents

Helium gas (Argyle Welding Supply, Albuquerque, NM) was used as received for degassing the mobile phase and other solutions. Acetonitrile and methanol, both HPLC grade were obtained from Sigma. 2,4-dinitrophenol, 2,4,6-trichlorophenol, pentachlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, 4-nitrophenol, p-cresol, o-cresol, 2-chlorophenol, 2-nitrophenol, and p-chlorophenol were all obtained from Sigma. 2,4-dimethylphenol, creosol, 4,6-dinitro-o-cresol, were from Aldrich, and phenol was from Fisher.

Tyrosinase enzyme (E.C. 1.14.18.1., T-7755), 4,400 Units/mg solid, was obtained from Sigma. Mineral oil and graphite powder were obtained from Aldrich and Fisher, respectively. Sodium phosphate buffer (PB, 0.05M) was adjusted to pH 6.5. HPLC grade water was used throughout the experiment. Environmental water samples were obtained from: Oak Ridge Creek Water, Tennessee, and Hanford Ground Water, Washington State. Membrane filters include: nylon membranes and microfilters, 0.2μm pore size, 47mm. All electrodes and electroanalizers were obtained from Bioanalytical Systems Inc.

High Performance Liquid Chromatography

All standard solutions were prepared in the mobile phase. All mobile phases, samples and solutions were filtered and degassed prior to use. The most frequently used mobile phase was acetonitrile (25:75) PB, pH 6.5, 0.05M. All experiments were performed on a reverse phase high performance liquid chromatography system (Model BAS 480, Bioanalytical Systems Inc., Lafayette, IN). A dual reciprocating pump PM-80 (BAS), complete with a dual electrochemical detector LC-4C (BAS), was used with BAS thin layer electrochemical cell in parallel mode. A flow rate of 0.5ml/min was used throughout the experiment. The plain carbon paste electrode (CPE) was operated at +1.2V, and the enzyme electrode, tyrosinase (3.0%) CPE, at -0.2V. Injections were made manually through a 20μl Reodyne loop injection valve, onto a BAS-C_{18} column (BAS, MF6213), ODS-3, 3μm, 3.2x100mm. Chromatograms were recorded on an Omniscribe strip chart dual pen recorder (Houston Instruments).

Electrode Design and Preparation

The thin layer cell (Figure 3-1) was operated in parallel mode. One cavity contained the plain carbon paste electrode (CPE) prepared using a 60:40 graphite powder:mineral oil composition, which was mixed thoroughly, and then packed into the electrode cavity. The second cavity was used for the enzyme electrode and was prepared using carbon paste, with a tyrosinase content of 3% (w/w). Both electrodes were carefully polished using ultra high purity weighing paper, taking care not to cross contaminate the surfaces. The electrode cell was then placed into its housing unit, the carrier buffer flow initiated, and the appropriate potentials applied: +1.20V for the plain CPE, and -0.20V for tyro-sinase (3.0%) CPE. Current was monitored, using the strip chart reorder and the baselines were allowed to stabilize before any analysis was performed.

Sample Analysis

Quantities of water samples from Hanford Ground Water (pH 7.8), WA, and Oak Ridge Creek Water (pH 6.6), TN, were spiked with known concentrations of phenol compounds. The samples were filtered and degassed, and without further pretreatment, injected onto the column. A control was also performed by injecting unspiked water samples. Calibration curves were also prepared, and the percentage recovery for the samples determined.

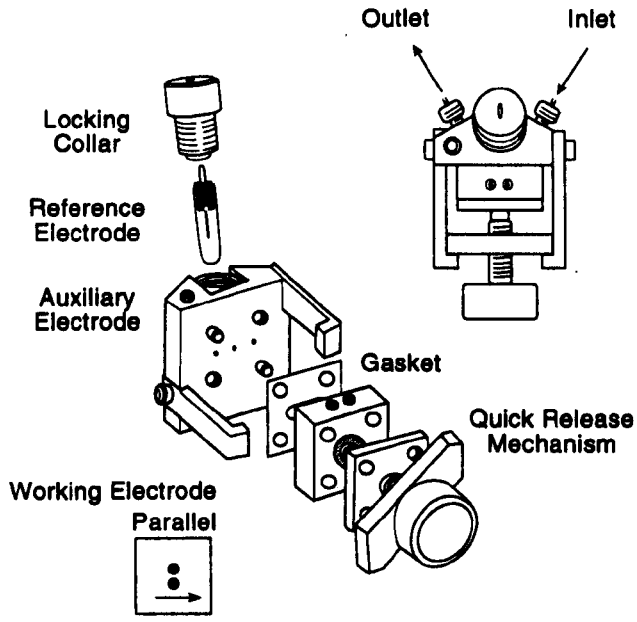

Figure 3-1. Schematic for (dual carbon paste electrode) electrochemical flow cell detector.

Chapter 4
Results and Discussion

Preliminary experiments were conducted for optimizing the enzyme-electrode and mobile-phase compositions, as well as experimental variables such as the flow rate and operating potentials. An enzyme loading of 3%w (in the carbon paste) coupled to a mobile phase containing 25% acetonitrile and 75% phosphate buffer (0.05M, pH 6.5) were thus selected for subsequent work, along with operating potentials of -0.2 and +1.2V for the enzyme- and plain electrodes, respectively.

Figure 4-1 displays the dual-electrode liquid chromatography with electrochemical detection (LCEC) response for a mixture containing 0.5mM 2,4-dinitrophenol, phenol, p-nitrophenol and o-cresol (peaks 1, 2, 3, and 4, respectively). Both the enzyme (A) and the plain (B) electrodes respond favorably, with high signal-to-noise characteristics, to these phenolic pollutants. (Notice the different current scales.) These data demonstrate also the compatibility of the tyrosinase electrode with the partially organic mobile phase; the latter is expected from the known organic-phase activity of tyrosinase (4).

Table 4-1 summarizes the retention times, detection limits and enzyme/plain-electrodes peak ratios for 15 environmentally important phenolic contaminants. Most species, with the exception of 2,4,5-trichlorophenol, are eluted within less than 25 minutes. The enzyme electrode detectors offer extremely low detection limits; these range from 1.18ng (for phenol) to ca. 400ng (for pentachlorophenol). The peak-current ratios (enzyme/bare electrodes) range from 0.76 (for 4,6-dinitro-o-cresol) to 1.10 (for 2,4-dichlorophenol). Such different peak ratios greatly enhances the information content of the LCEC response. A typical dual-electrode response for a mixture of these 15 phenolic pollutants is displayed in Figure 4-2.

Peak #	Compound (0.5mM)
1	2,4-dinitrophenol
2	Phenol
3	p-nitrophenol
4	o-cresol

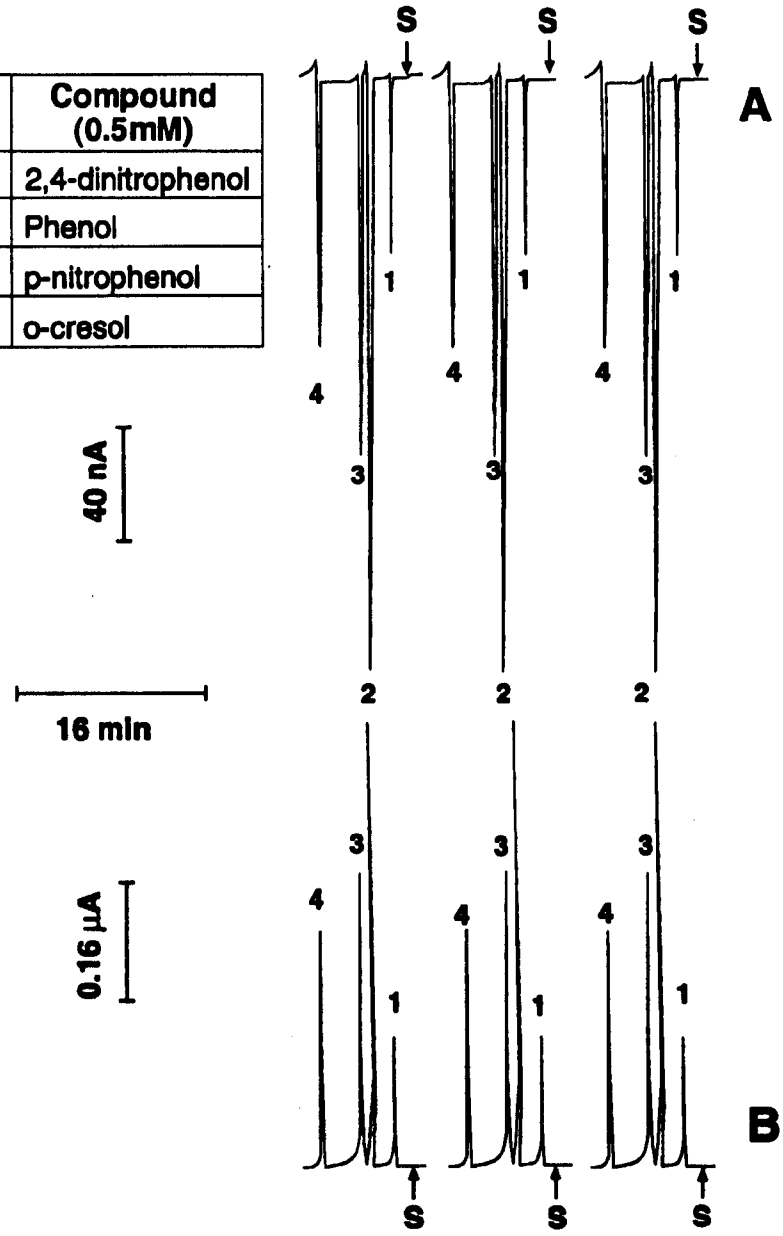

Figure 4-1. **Preliminary results of phenol analysis using reverse phase HPLC-dual electrode. Chromatograms of four-component phenol mixture at pH 6.5 under optimum mobile phase composition, acetonitrile (27:75) PBS, 0.05M. Flow rate, 0.5ml/min. (A) Enzyme electrode at -0.2V; (B) plain carbon electrode, +1.2 V. See text for other conditions. Conditions: *Mobile Phase*: Acetonitrile: 0.05M PBS Buffer pH 6.5 (25:75), *Flow Rate*: 0.5ml/min, *Injection Loop*: 20µl, Column: BAS C_{18}, *Detector*: Dual Electroder (A: tyrosinase (3.0%) CPE, -0.2V; B: Plain CPE, +1.2V).**

EPA Project

Conditions

Mobile Phase: 25:75 Acetonitrile:PBS pH 6.5, 0.05M
Flow Rate: 0.5ml/min
Column: BAS C-18
Loop Size: 20μl

Table 4-1. Retention times, detection limits and peak ratios of a mixture of 15 phenolic compounds.

Peak	Compound	Retention Time (min)	Detection Limit* (ng)	Peak Ratios**
1	2,4-Dinitrophenol	1.92	6.90	0.78
2	4,6-Dinitro-o-cresol	2.40	6.19	0.76
3	Phenol	2.80	1.18	0.80
4	4-Nitrophenol	3.52	2.17	0.85
5	p-cresol	6.00	6.76	0.84
6	o-cresol	6.80	3.38	0.86
7	creosol	6.80	3.05	0.80
8	2-Chlorophenol	7.28	2.19	0.85
9	2-Nitrophenol	7.44	8.70	0.85
10	p-Chlorophenol	9.40	2.17	0.88
11	2,4-Dimethylphenol	11.80	9.16	0.85
12	2,4,6-Trichlorophenol	16.40	98.70	0.95
13	Pentachlorophenol	18.10	399.45	0.97
14	2,4-Dichlorophenol	22.40	3.44	1.10
15	2,4,5-Trichlorophenol	46.20	74.03	0.97

* Detection limits were obtained at signal-to-noise of 3, and calculated for tyrosinase (3.0%) CPE only.

** Peak ratios calculated, $(I_{tyrosinase(3.0\%)CPE}/I_{CPE})*10$.

Figures 4-3 and 4-4 display calibration plots obtained at the tyrosinase-based flow detector for 2,4-dinitrophenol, phenol, p-nitrophenol and o-cresol. All four phenolic compounds display good linearity over the entire concentration range (0 - 25μM) examined. The slopes of these plots (i.e., the sensitivity) correspond to 0.381-(o-cresol), 0.424-(p-nitrophenol), 0.449-(2,4-dinitrophenol) and 0.627-(phenol) nA/μM (correlation coefficients, 0.999). Similar calibration plots for the same compounds over a wider concentration range (10 - 100μM) resulted in some curvature above 30μM (not shown).

Peak #	Compound
1	2,4-Dinitrophenol
2	4,6-Dinitro-o-cresol
3	Phenol
4	4-Nitrophenol
5	p-cresol
6	o-cresol
7	creosol
8	2-Chlorophenol
9	2-Nitrophenol
10	P-Chlorophenol
11	2,4-Dimethylphenol
12	2,4,6-Trichlorophenol
13	Pentachlorophenol
14	2,4-Dichlorophenol
15	2,4,5-Trichlorophenol

A = Tyrosinase (3.0%) CPE @ -0.20V

B = Plain CPE @ +1.20V

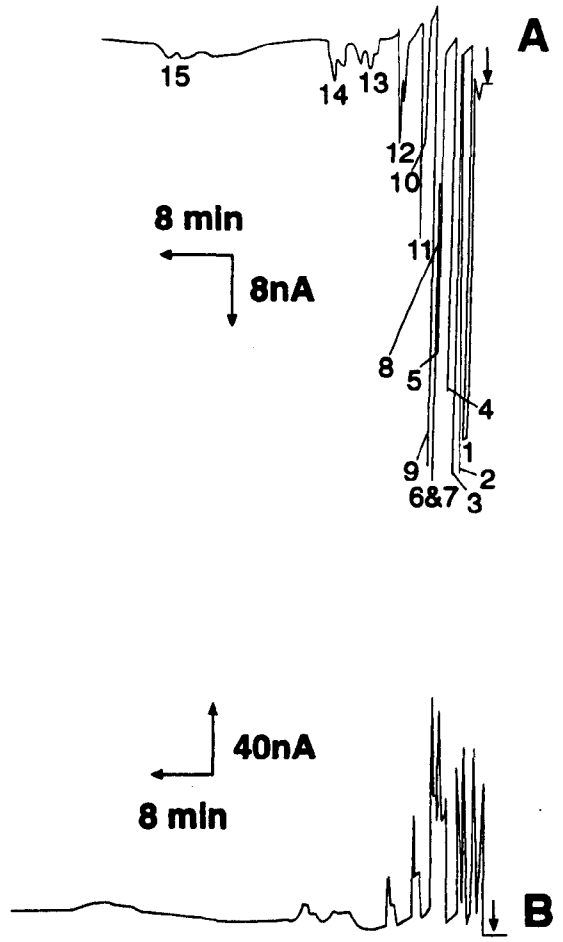

Figure 4-2. **Chromatograms of 15-component phenol mixture at pH 6.5, under optimum experimental conditions. Conditions: *Mobile Phase*: 25:75 Acetonitrile: PBS pH 6.5, 0.05M, *Flow Rate*: 0.5 ml/min, Column: BAS C-18, Loop Size: 20μl, *Sample*: 0.1 mM solution phenol compounds, *Detector*: Dual Electrochemical Detector in parallel (tyrosinase (3.0%) CPE at -0.2V and Plain CPE at +1.2V).**

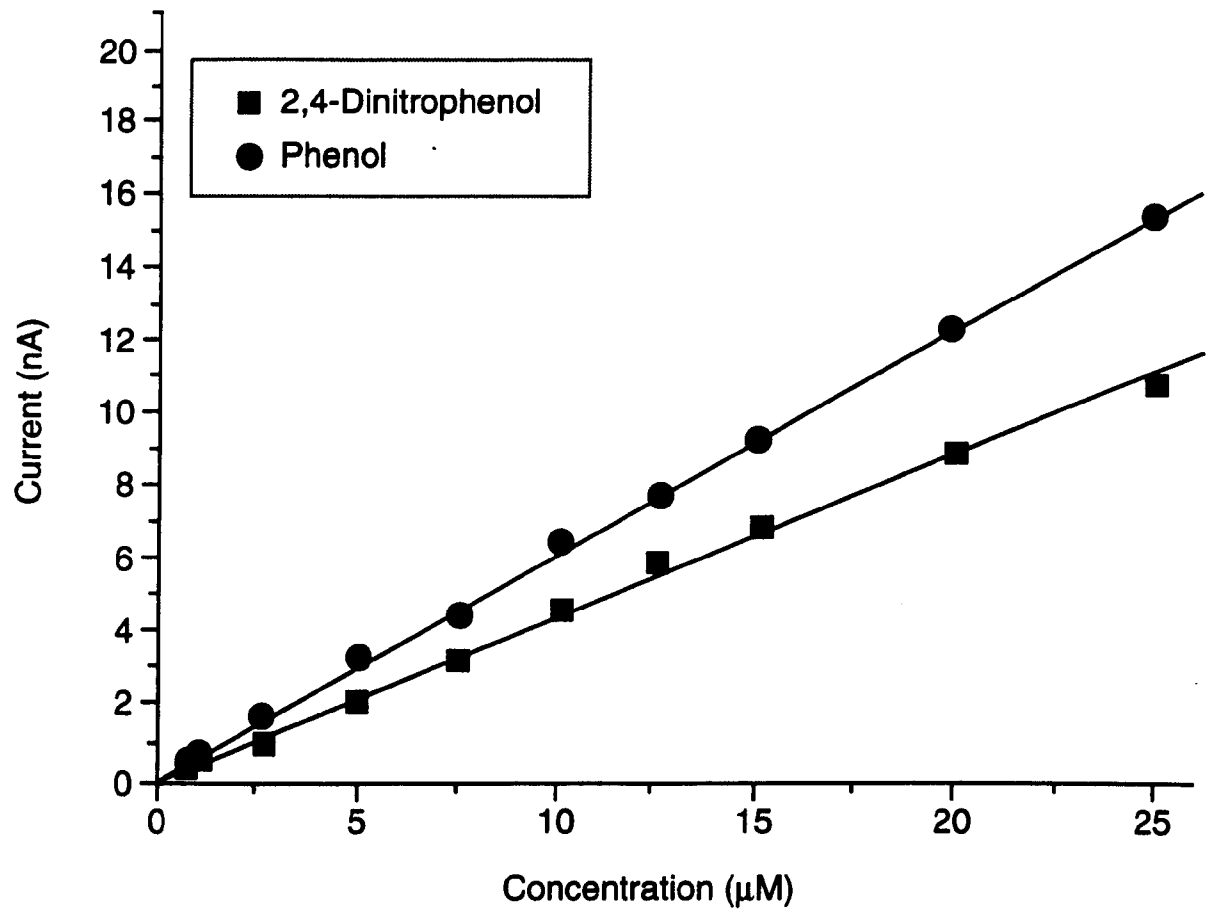

Figure 4-3. Typical calibration plots for 2,4-dinitrophenol and phenol at tyrosinase (3.0%) carbon paste electrode, using optimal experimental conditions.

Application of the new LCEC protocol for the analysis of spiked environmental samples is shown in Figure 4-5. Both the groundwater (c) and creek water (d), from the Hanford and Oak-Ridge locations, respectively, offer convenient quantitation of the four spiked phenols (at the 12.5μM level). No other peaks are observed at both electrodes, reflecting the high specifity of the method using these matrices. Recovery values (at the enzyme electrode) for these phenols in the groundwater sample range from 107 to 119%, while in the creek water sample from 103 to 114%. The peak-current ratio values (I_{enz}/I_{bare}), using the groundwater and creek water samples are 0.99 and 0.96 (p-nitrophenol), 0.78 and 0.78 (2,4-dintrophenol), 0.84 and 0.93 (o-cresol) and 0.87 and 0.79 (phenol), respectively.

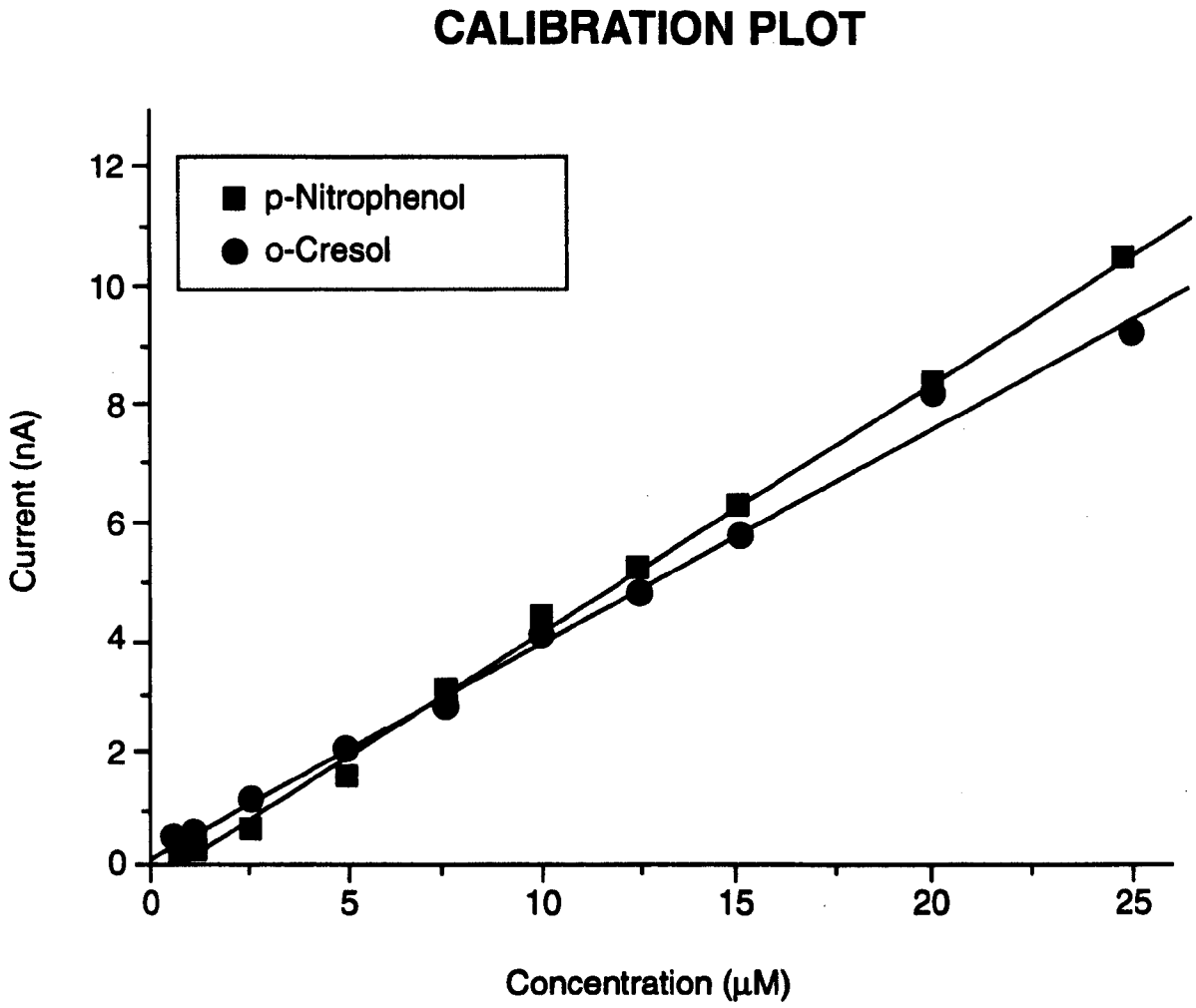

Figure 4-4. Typical calibration plots for p-nitrophenol and o-cresol at tyrosinase (3.0%) carbon paste electrode, using optimal experimental conditions.

Peak #	Compound
1	2-dinitrophenol
2	Phenol
3	p-nitrophenol
4	o-cresol

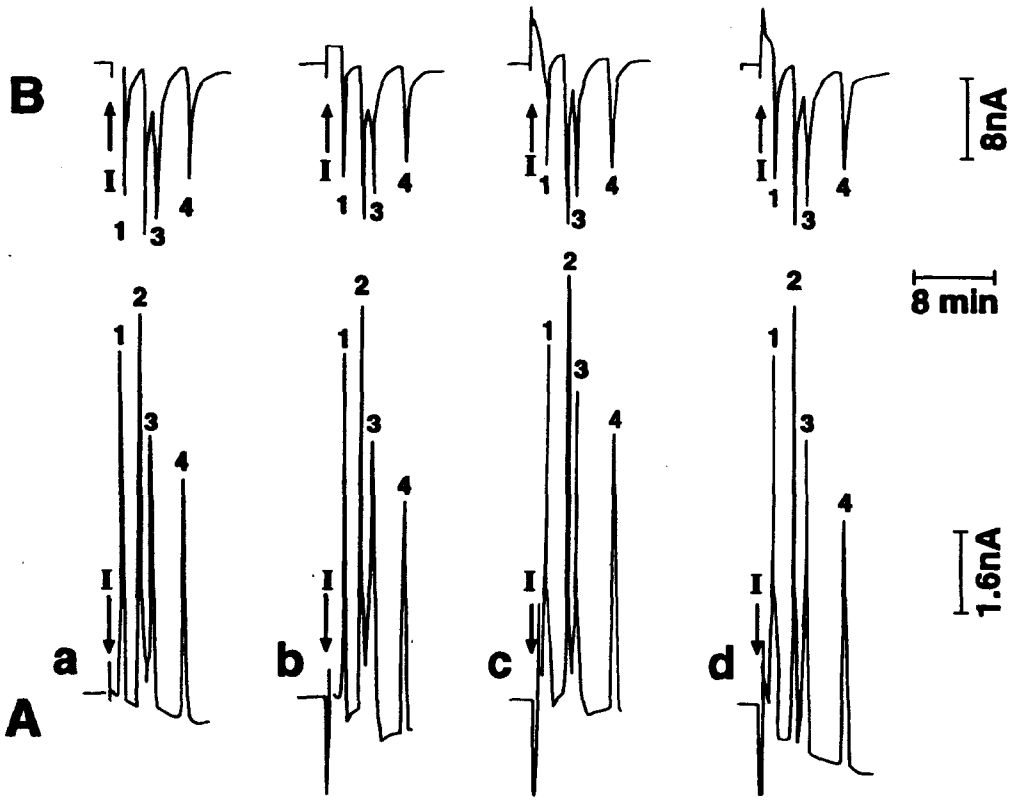

Figure 4-5. Sample analyses. Typical chromatograms of real water samples spiked with a 12.5μM four-component phenol mixture under optimum experimental conditions. Conditions: *Mobile Phase*: 25:75 Acetonitrile: PBS (pH 6.5, 0.05M), *Flow Rate*: 0.5 ml/min, *Column*: BAS C_{18}, *Loop Size*: 20μl, Detector: Dual Electrochemical Detector in parallel (tyrosinase (3.0%) CPE, -0.2V; Plain CPE, +1.2V), Sample: a: Typical injection of 12.5μM phenol mixture onto column, b: 50% Hanford Ground Water, WS, spiked with 12.5 μM phenol mixture, c: Hanford Ground Water, WS, spiked with 12.5 μM phenol mixture, d: Oak Ridge Creek Water, TN, spiked with 12.5 μM phenol mixture, I: Injecting sample, A: tyrosinase CPE; B: Plain CPE.

Chapter 5
Conclusions and Recommendation

Overall, this project has demonstrated that enzyme-based biosensors for phenols are compatible with the monitoring of these pollutants in liquid-chromatographic effluents. The tyrosinase-based detector enhances the information content of chromatographic measurements of phenolic contaminants, and facilitates their monitoring in relevant environmental samples. Such a detector can be employed also in a flow-injection system, that would provide a rapid screening for the "total" phenol content. Indeed, one can switch between the flow injection and liquid-chromatographic systems (using an appropriate valve) to obtain the rapid screening first, and whenever needed (for elevated levels) switch to the more detailed chromatographic analysis.

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