the heart tissue at 420–350% over that of LDH activity (assayed in our laboratory). We found that it was impossible to separate completely the LDH and MDH activities on the affinity adsorbent BM 1. Applying the affinity-purified fraction with LDH activity on the anion exchanger, the contaminating mitochondrial MDH passed through, whereas cytoplasmic MDH bound the adsorbent and was removed with 100 mM KCl, prior to adsorbed LDH being eluted with 200 mM KCl.

Quality-control tests on L-lactate dehydrogenase purified by the above two-step procedure showed a specific activity 490 u/mg (25°C) and the following contaminating activities: MDH,

0.01%; pyruvate kinase and glutamic–oxaloacetic transaminase, not detectable. The purified enzyme showed one major band on SDS-PAGE (Fig. 3). The specific activity and contaminating activities of commercial bovine heart LDH, suitable for analytical purposes, have been quoted as follows: 250 u/mg (25°C); MDH, less than 0.05%; and pyruvate kinase, less than 0.01%. Further, our procedure compares favourably with that employing the triazine dye Procion Scarlet MX-G immobilized on a cellulose support via a diaminohexyl spacer [41,42]. The latter approach led to enzyme of specific activity 200 u/mg [41]. Consequently, the present method provides a simple and effective way for preparing, in two steps and with a good yield, L-lactate dehydrogenase suitable for analytical purposes.
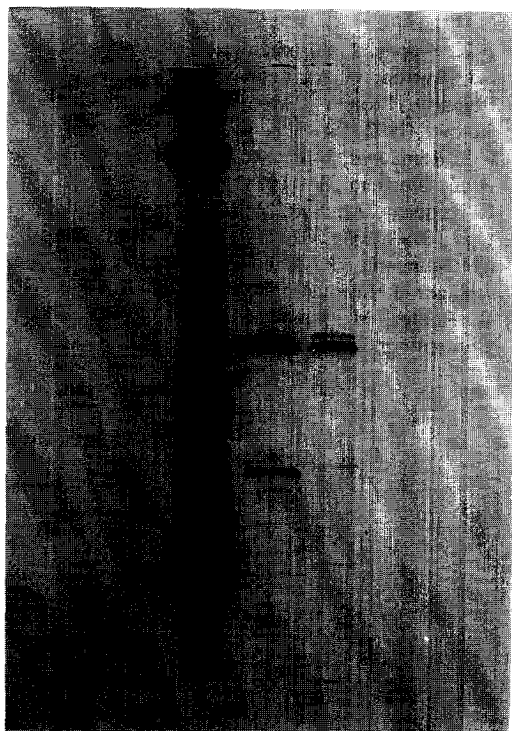
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Fig. 3. SDS-PAGE of LDH. SDS-PAGE was performed on a 1.5 mm thick vertical slab gel (14 × 16 cm) containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The protein bands were stained with Coomassie Bue R-250. Left lane, bovine heart extract; middle lane, LDH purified from the biomimetic dye affinity column (after step 1, Table 4); right lane, LDH purified from the anion-exchange column (after steps 1 and 2, Table 4).

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High-performance liquid chromatographic separation and detection of phenols using 2-(9-anthrylethyl) chloroformate as a fluorophoric derivatizing reagent

Walter J. Landzettel[a], Kelly J. Hargis[a], Jason B. Caboot[a], Kent L. Adkins[a], Timothy G. Strein[a], Hans Veening[a,*], Hans-Dieter Becker[b]

[a]Department of Chemistry, Bucknell University, Lewisburg, PA 17837, USA
[b]Department of Organic Chemistry, Chalmers University of Technology and University of Gothenburg, S-412 96 Gothenburg, Sweden

Abstract

This paper describes the use of 2-(9-anthrylethyl) chloroformate (AEOC) as a sensitive and convenient pre-column, fluorophoric derivatizing reagent for the separation and detection of phenol (P), p-methylphenol (PMP), 3,4-dimethylphenol (DMP), and 4-tert.-butylphenol (BP) using reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. Experiments were carried out to determine maximum fluorescence excitation and emission wavelengths, optimum derivatization pH, efficient gradient programming, calibration curves, minimum detection limits, and stabilities for AEOC-derivatized phenols. The method was applied to several industrial waste water samples from Central Pennsylvania. The minimum detection limits for injected phenol samples ranged from 7 to 10 nM, or 9, 10, 12 and 11 pg for P, PMP, DMP and BP, respectively. This corresponded to concentrations of phenols in the original waste water samples of 56 to 80 nM.

1. Introduction

Analytical procedures for phenols are used in a wide range of applications. Phenols may occur in domestic and industrial waste waters, natural waters, and municipal water supplies [1]. Phenols are also used as starting material for several food preservatives (butylated hydroxy-toluene and hydroxyanisole), herbicides, antiseptics (hexachlorophene), explosives, and polymers [2]. Phenols, such as vinylphenol, vinylguaiacol, ethylphenol, and ethylguaiacol, are important components in wines [3]. Phenol and p-cresol have also been found to be present in uremic serum samples collected from patients suffering from renal dysfunction [4]. Previous high-performance liquid chromatography (HPLC) detection methods for phenols have included UV absorption and photodiode-array techniques [5], fluorescence [4] and electrochemical methods [6,7]. Li and Kemp [8] developed a reductive, amperometric HPLC method to determine phenols between concentrations of 10^{-7} and 10^{-4} M in aqueous samples using pre-column derivatization with 4-aminoantipyrine. While the method is selective for phenols, it also requires

* Corresponding author.

an extraction step during derivatization. Imai et al. [9] reported a pre-column derivatizing reagent, 4-(N-chloroformylmethyl-N-methyl)-amino-7-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole, to determine phenols, alcohols, amines, and thiols in the fmol range. The derivatization step did require a catalyst. Zheng et al. [10] have described an HPLC method to detect 80 to 160 fmol of phenols by pre-column derivatization with 4-(2-phthalimidyl)benzoyl chloride and fluorescence detection. A pre-column HPLC derivatization procedure for phenols with dansyl chloride and imidazole (catalyst) leading to chemiluminescence detection was reported by Fu [11]. The detection limits were 1.2 to 2.2 pg for phenol and several alkyl phenols.

Fluorophoric chloroformates, such as 9-fluorenylmethyl chloroformate (FMOC), 2-(9-anthryl)ethyl chloroformate (AEOC), and 2-(1-pyrenylethyl) chloroformate (PEOC) have been used successfully as pre-column derivatizing agents in HPLC for the determination of analytes containing primary amine groups. The chloroformate functionality reacts with primary and secondary amines to form fluorescent carbamates. This reaction has been utilized successfully in pre-column HPLC derivatization procedures for the determination and detection of amino acids [12–14] and polyamines [15]. Recently, we reported AEOC and PEOC to be effective fluorophoric, pre-column HPLC reagents for the determination of biogenic polyamines [16,17]. We now report the use of AEOC as an HPLC pre-column derivatizing reagent for phenol (P), p-methylphenol (PMP), 3,4-dimethyl-phenol (DMP), and 4-tert.-butylphenol (BP). AEOC, like other chloroformates, reacts with phenols to form fluorescent, aromatic carbonates. Excess AEOC undergoes hydrolysis in aqueous media to form 2-(9-anthryl)ethanol (AEOH) which may react further with AEOC to give bis(2-(9-anthryl)ethyl) carbonate (BAEC), as shown in Fig. 1, reactions 1–3. The method reported here utilizes a newly synthesized fluorophoric chloroformate (AEOC), which we previously used successfully for pre-column derivatization of polyamines determined by HPLC [16]. In this investigation we have demonstrated the
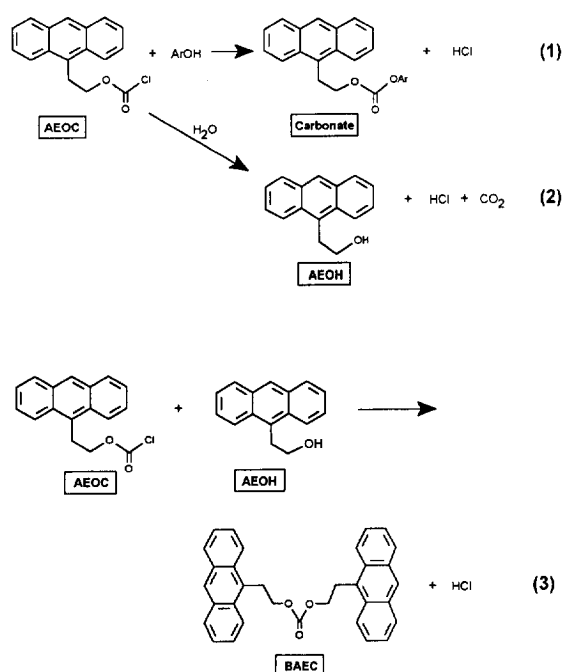

Fig. 1. Reaction of AEOC with phenols to form fluorescent carbonates (reaction 1) and hydrolysis of AEOC in aqueous media to form AEOH (reaction 2), which may react with AEOC to give BAEC (reaction 3).

utility of AEOC, a as a versatile, fluorophoric, pre-column HPLC derivatizing reagent for determining traces of phenols contained in environmentally significant samples, such as industrial waste water.

2. Experimental

2.1. Apparatus

The chromatographic system was a Spectra-Physics SP-8800 ternary solvent delivery system (Spectra-Physics, San Jose, CA, USA) equipped with a Model 7010 Rheodyne injection valve and a 10-μl sample loop. Fluorescence detection was achieved with a Model 750 BX McPherson instrument (McPherson, Acton, MA, USA). An excitation wavelength of 256 nm (5 nm bandwidth) and a 418-nm low-end cutoff emission filter were used. The sensitivity range was set at 0.003 μA with a time constant of 0.5 s and low

suppression background. Fluorescence spectra were obtained using a Perkin-Elmer LS-50 Luminescence Spectrometer (Perkin Elmer, Oak Brook, IL, USA). A Hewlett-Packard 3396A integrator (Hewlett-Packard, Wilmington, DE, USA) was used for peak integration and for recording all chromatograms.

The analytical LC column was a Hewlett-Packard LiChrospher 100 RP-18, 125×4.0 mm I.D., packed with 5-μm particles. Separations were carried out at a flow-rate of 0.75 ml/min using a binary gradient of 70% acetonitrile, 30% deionized water (pH 6.1) programmed to 100% acetonitrile in 10 min and an isocratic post-time of 10 min. The column inlet pressure was 480 p.s.i. (3309.48 kPa). The column temperature was ambient.

2.2. Chemicals and reagents

Phenol (P), p-methylphenol (PMP), 3,4-dimethylphenol (DMP), and 4-$tert.$-butylphenol (BP) were obtained from Aldrich Chemical (Milwaukee, WI, USA) and were used without further purification. HPLC-grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade water was generated in-house using a water purification system manufactured by Industrial Water Technology (No. Attleboro, MA, USA). Reagent-grade hydrochloric acid, sodium hydroxide, and sodium borate were used to prepare buffer solutions and were obtained from Fisher Scientific. AEOC was synthesized as previously described [18–20]. Since AEOC solutions have been found to undergo reactions upon exposure to laboratory light [18–20], reagent stock solutions were kept in light-protected containers and stored under refrigeration when not in use.

2.3. Derivatization procedure

Derivatization of phenol standards was accomplished by adding 500 μl of 2.59 μM AEOC in HPLC-grade acetonitrile to 100 μl of a standard mixture containing P, PMP, DMP, and BP and 200 μl of pH 9.6 aqueous borate buffer (0.025 M). The derivatization solution was allowed to

stand for 35 min at 43°C to permit completion of the reaction, and a 10-μl sample was then injected. Industrial waste water samples were obtained from Wilson Testing Laboratories (Shamokin, PA, USA) and were filtered through a 0.2-μm filter to remove particulate matter. The water sample (100 μl) was then derivatized for 35 min at 43°C after adding 500 μl of 2.59 μM AEOC (in acetonitrile) and 200 μl of pH 9.6 borate buffer.

3. Results and discussion

The fluorescence excitation and emission spectra for five collected fractions of AEOC-derivatized phenol in acetonitrile (1.0 μg of phenol with excess AEOC injected) are shown in Fig. 2. The emission spectrum was scanned at $\lambda_{exc} = 256$ nm and the excitation spectrum at $\lambda_{em} = 389$ nm. Prominent excitation and emission maxima were observed at 256, 389, 412, and 436 nm, respectively. Very weak excitation bands were observed
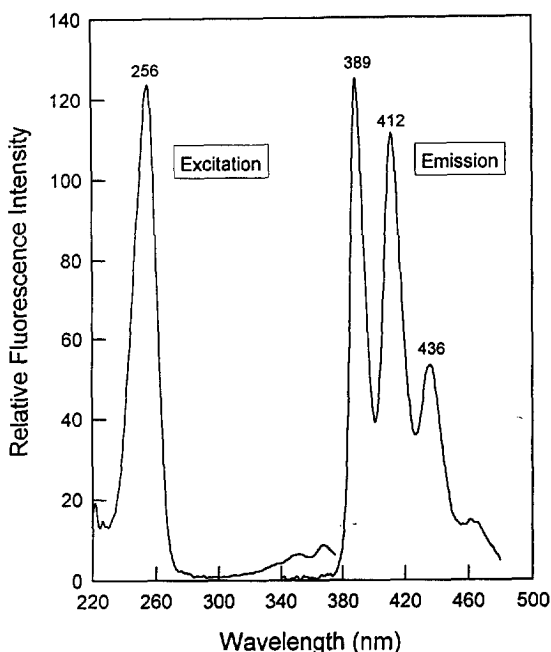

Fig. 2. Fluorescence excitation and emission spectra of eluted AEOC-derivatized phenol in CH_3CN (1.0 μg of phenol with excess AEOC injected).

at 352 and 367 nm, as shown in Fig. 2. (Scanning the excitation spectrum at $\lambda_{em} = 412$ nm yielded the same excitation spectrum.) The spectra obtained were very similar to those of other derivatized phenols and to the spectra reported previously for AEOC-, AEOH-, and AEOC-derivatized polyamines [16]. Based on the spectral data, HPLC detection of AEOC-derivatized phenols was optimized by using an excitation wavelength of 256 nm and a 418-nm low-cutoff emission filter. Use of a 390-nm low-end cutoff filter was investigated and found to be much less sensitive than the 418-nm filter.

Complete derivatization of phenols with AEOC was found to require 35 min at 43°C. The reaction of AEOC with phenols was found to be pH dependent. The optimum reaction pH was determined by derivatizing each of the four phenols at pH values ranging from 7.50 to 10.00 and measuring the fluorescence response ($\lambda_{exc} = 256$ nm; $\lambda_{em} > 418$ nm) for each eluted analyte as a function of pH. The results, shown in Fig. 3, indicate three maxima, at ca. pH 8.4, 8.9 and 9.6. It is clear that the pH dependence of the
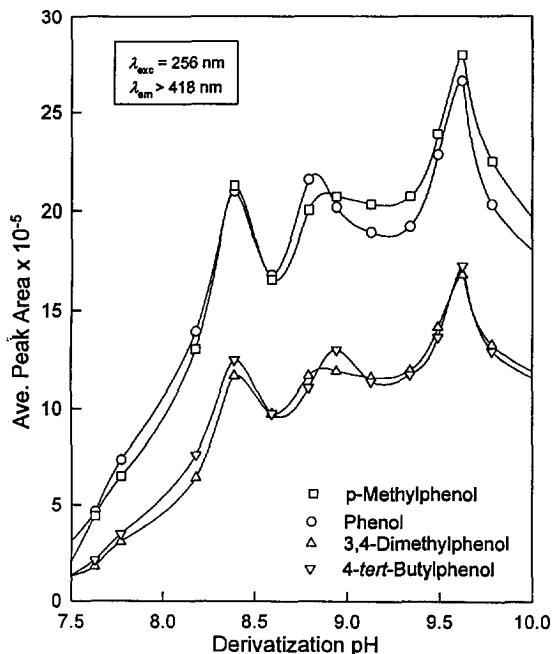
derivatization reaction is not straightforward; however, these results permitted the selection of an optimum derivatization pH of 9.60.

A chromatogram for the separation of a standard mixture of the four AEOC-derivatized phenols (P, PMP, DMP, and BP) is shown in Fig. 4. The identity of the BP peak at 12.32 min was confirmed by comparison of its retention time to that of an authentic sample of AEOC-derivatized 4-tert.-butylphenol, synthesized independently. The identity of the by-product peaks, AEOH and BAEC, was confirmed similarly. The reaction of AEOC with AEOH to form the carbonate (BAEC) is illustrated in Fig. 1, reac-
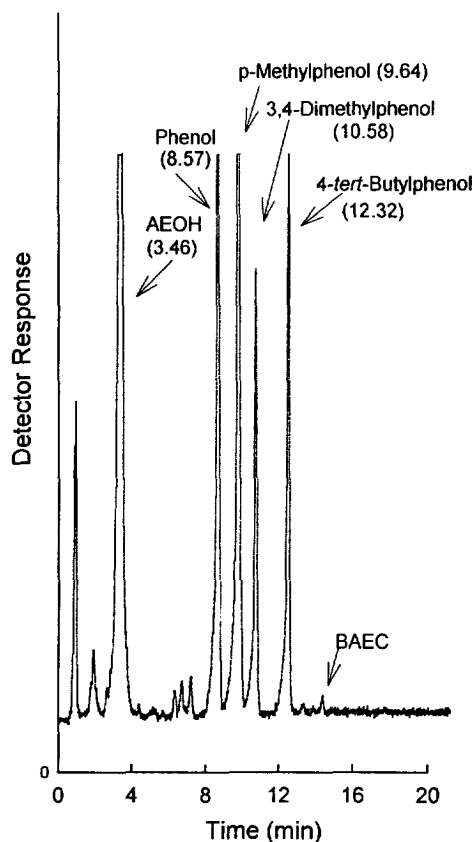

Fig. 4. Chromatogram for AEOC-derivatized phenol standards (0.2 ng of each phenol injected). Flow-rate: 1.0 ml/min, temperature: ambient, column: LiChrospher 100 RP-18, 100×10 mm, mobile phase: 70% CH_3CN–30% deionized H_2O (pH 6.1) to 100% CH_3CN in 10 min, isocratic post-time: 10 min, detection: fluorescence, $\lambda_{exc} = 256$ nm, $\lambda_{em} > 418$ nm.

Fig. 3. Detector response versus derivatization pH for eluted AEOC–phenol derivatives.

tion 3. Neither of these by-products interfered in the separation; in fact, BAEC was found to be a useful relative retention time internal standard. Although, the retention times varied slightly from run to run, the retention times relative to BAEC were consistent. Under the gradient conditions used, the four derivatized phenols were completely resolved from each other and from reaction by-products.

A plot of corrected peak area versus nano-grams of phenol injected is shown in Fig. 5. Data points were obtained by individually derivatizing solutions containing successively lower concen-trations of each of the phenols prior to chroma-tography. The original plots exhibited significant curvature due to non-linear detector behavior at low concentrations. The data were re-plotted according to the method of Dorschel et al. [21] in order to show the actual response more clearly. In order to confirm completeness of derivatization, a second experiment was per-formed in which the calibration curve was de-termined by injecting increasingly smaller con-centrations of already derivatized solutions of the phenols. The calibration curve obtained was identical to that shown in Fig. 5.

The limits of detection for this procedure were determined for each of the four phenols. This was accomplished by setting the detector sen-sitivity at the maximum range of 0.003 μA and individually derivatizing successively diminishing quantities of each of the diluted phenols prior to injection. Each detection limit was obtained by recording the smallest amount of phenol that still produced a peak at a signal-to-noise ratio of 3:1. The minimum detection limits for injected phenol samples ranged from 7 to 10 nM, or 9, 10, 12, and 11 pg for P, PMP, DMP, and BP, respectively. This corresponded to concentra-tions of phenols in the original waste water samples of 56 to 80 nM. A chromatogram illustrating the separation of a mixture of ca. 0.01 ng of each derivatized phenol injected (i.e., approximately the detection limit) is shown in Fig. 6.

In order to examine the stability of the AEOC–phenol derivatives, derivatized phenol solutions were allowed to stand at room tem-
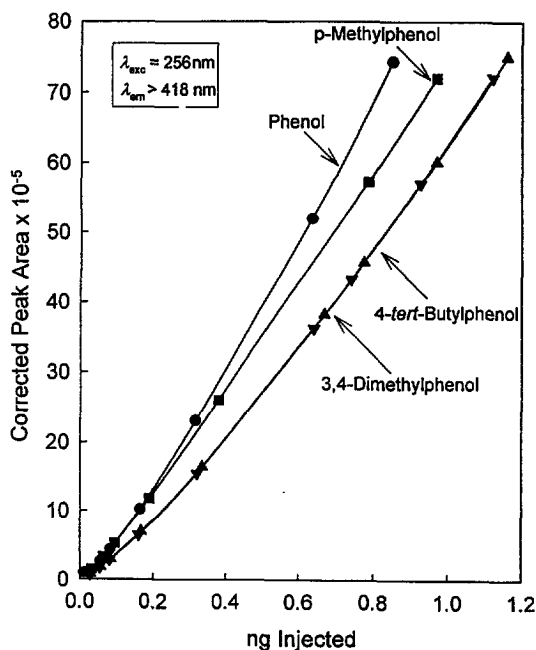

Fig. 5. Corrected peak area versus ng of analyte injected. (Experimental data replotted according to Ref. [21].)
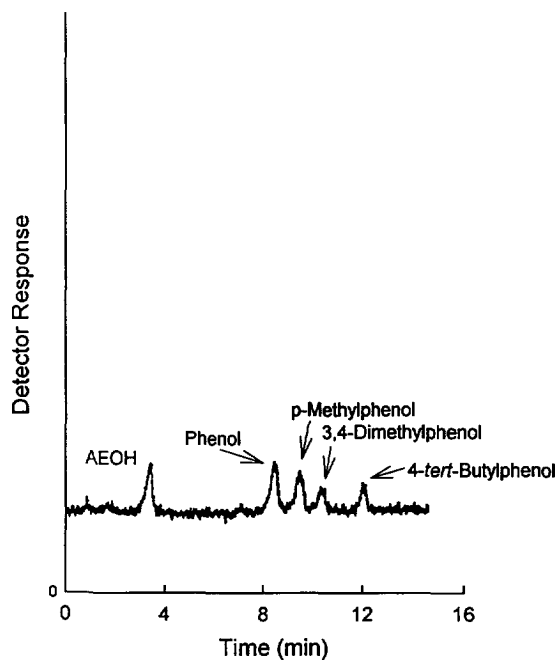

Fig. 6. Detection limits for AEOC-derivatized phenols (ca. 0.01 ng injected). Conditions the same as in Fig. 4.