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PHOTOOXYGENATION-CHEMILUMINESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTOR FOR THE DETERMINATION OF ALIPHATIC ALCOHOLS, ALDEHYDES, ETHERS AND SACCHARIDES

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SUMMARY

A novel chemiluminescence high-performance liquid chromatographic (HPLC) detector capable of quantitating many oxygen-containing compounds that cannot be determined by either absorption or fluorescence has been developed in our laboratory. The photooxygenation chemiluminescence (POCL) detector functions by first oxygenating the analytes —aliphatic alcohols, aldehydes, ethers and saccharides— in a photochemical reaction to produce H_2O_2. The H_2O_2 is then quantitated in the cobalt(II)–luminol chemiluminescence reaction. Since the photochemical reaction is sensitized by anthraquinonedisulfonate, the analytes themselves need not absorb light. The sensitivity of the detector varies for each analyte; however, the detection limits of most analytes are in the low microgram range. This paper demonstrates the use of this detector coupled to HPLC and presents optimization studies of the photochemistry and the chemiluminescence.

INTRODUCTION

Great interest has recently been focused on the use of chemiluminescence (CL) for liquid phase quantitative analysis. Although liquid phase CL has been known for more than a century, it has only recently seen application in analytical systems[1]. Current interest in analytical applications of chemiluminescence stems from its many valuable properties —simple and inexpensive instrumentation required, short analysis times, and good sensitivity.

Previous liquid phase CL techniques have most often employed the CL reaction of luminol, lucigenin or bis(trichlorophenyl) oxalate (TCPO). Luminol and lucigenin have been used successfully as titration indicators in acid–base titrations of opaque substances —milk and red wine[2–4]— while luminol alone has been employed in the trace analysis of certain transition metals —Co(II), Cu(II), Ni(II), and Fe(II)— which act as catalysts for the CL reaction[5]. In addition, luminol has been used in copper(II)-catalyzed reactions to determine H_2O_2 in atmospheric[6] samples. Furthermore, enzyme-induced CL techniques have been employed with luminol and TCPO[7].

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In these techniques H_2O_2 is generated enzymatically from particular analytes followed by a catalyzed luminol or TCPO reaction which quantitates the H_2O_2. Enzymatic CL techniques have been demonstrated for the determination of glucose, ATP, NADH and FMN[8].

Recently, CL detectors have been successfully combined with high-performance liquid chromatography (HPLC). Neary *et al.*[9] used luminol to analyze mixtures of Co(II) and Cu(II). Kobayashi and Imai[10] employed the TCPO–H_2O_2 system to quantitate femtomole quantities of dansyl amino acids. Birks and Kuge[11] used O_3 as the CL reagent in an aerosol spray and detected fluorescent compounds, olefins, certain nitrogen compounds and divalent sulfur compounds, Veazey and Nieman[12] constructed a lucigenin-base reductant system for the detection of ascorbic acid and other reducing compounds and Shoemaker and Birks[13] used $O_2(^1\Delta_g)$ for selective detection of chlorophylls *a*, *a'* and *b*.

The photooxygenation chemiluminescence (POCL) system described here first oxygenates the substrates to generate H_2O_2 in a sensitized photooxygenation reaction using a post-column photochemical reactor. The H_2O_2 produced is then quantitated by the cobalt(II)-catalyzed luminol CL reaction in a CL detection cell. Clearly the POCL detector bears resemblance to enzyme-based CL systems. In this study we have successfully combined the POCL system with HPLC for the analysis of aliphatic alcohols, aldehydes and ethers. In addition to these analytes, the POCL system can be employed in the detection of various saccharides.

THEORETICAL

The POCL detector functions by using an anthraquinone-sensitized photooxygenation reaction that procedures H_2O_2 during the oxidation of the analytes — alcohols, aldehydes, ethers and saccharides. Much of our understanding of these reactions is described by Bolland and Cooper[14] and Wells[15,16]. In this photooxygenation reaction the sensitizer anthraquinone-2,6-disulfonate (disodium salt) is excited to the triplet state by UV light near 365 nm. The absorption of light at this wavelength corresponds to an ($n \rightarrow \pi^*$) electronic transition and is followed by rapid intersystem crossing to the chemically reactive triplet state. The more strongly absorbing ($\pi \rightarrow \pi^*$) transition at shorter wavelengths is also effective at promoting photooxygenation[17]. Once formed, the excited sensitizer abstracts a hydrogen atom from the substrate producing two free radicals —one from the substrate and one from the sensitizer. These two free radicals then react with molecular oxygen to form HO_2 and RO_2 radicals, and subsequent reactions form H_2O_2. In the presence of primary and secondary alcohols a hydrogen atom is abstracted by the excited quinone from the carbon α to the functional group. This same route of attack is believed to be followed with aliphatic ethers; however, the exact mechanism which aldehydes follow is not discussed by Bolland and Cooper[14] or Wells[15,16]. Fig. 1 represents the most probable mechanism for the sensitized photooxygenation of ethanol in a neutral aqueous solution[15]. This sensitized photooxygenation reaction is termed a type I process by Gollnick[18] since it involves a hydrogen abstraction by the sensitizer. By contrast, in a type II process the excited sensitizer transfers energy to molecular oxygen to form $O_2(^1\Delta_g)$ which then oxidizes the substrates[19].

Once formed in the sensitized photooxygenation process, H_2O_2 can be easily

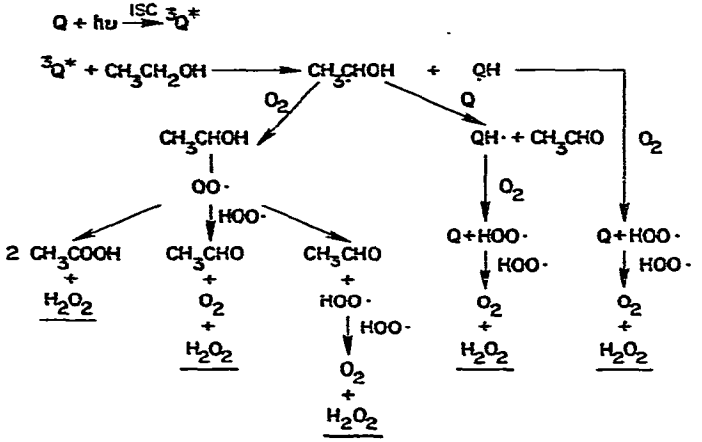


Fig. 1. Probable mechanism for the AQDS sensitized photooxygenation of ethanol in neutral aqueous solution. ISC = Intersystem crossing.

quantitated by the well-known reaction with luminol[20] shown in Fig. 2. The CL reaction is carried out in basic solution and in the presence of a transition metal catalyst —Co(II)— in order to increase the intensity of the CL reaction. Cobalt(II) was chosen since it is the most efficient catalyst for the hydrogen peroxide–luminol CL reaction[21]. The mechanism of the cobalt(II)-catalyzed reaction is believed to involve the formation of a Co(II)–peroxy complex which then oxidizes the luminol molecule, producing an electronically excited aminophthalate anion, the emitting species[21].

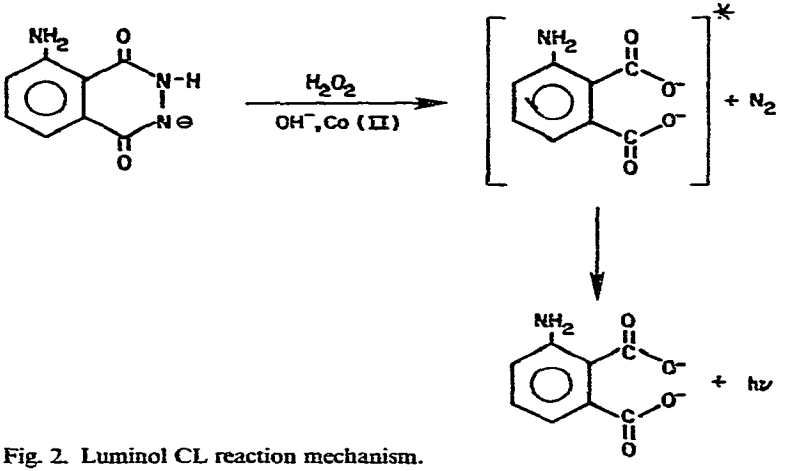


Fig. 2. Luminol CL reaction mechanism.

EXPERIMENTAL

Reagents

Anthraquinone-2,6-disulfonate, disodium salt (AQDS) (Aldrich, Milwaukee, WI, U.S.A.) was used without further purification to prepare a standard solution of 1

g per 500 ml (4.85×10^{-3} *M*), and when stored in a brown storage bottle was stable for many weeks. Luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione (Aldrich), was also used without further purification. A working solution of 2×10^{-4} *M* was made up in 0.1 *M* borate buffer at pH 10.9, and this working solution was also stable for many weeks when stored in a brown storage bottle. Cobalt nitrate, $Co(NO_3) \cdot 6H_2O$ (Fisher, Denver, CO, U.S.A.), was also used without further purification. Ninety percent H_2O_2 (F.M.C., San Jose, CA, U.S.A.) was diluted with acetonitrile to make the necessary H_2O_2 standards for the chemical yield experiments. These standards were stored at 5°C in the dark. Reagent grade acetonitrile (J. T. Baker, Phillipsburgh, NJ, U.S.A.) was used in the HPLC mobile phase. All aqueous solutions were made up in 18-MΩ deionized water.

Chromatographic apparatus

The chromatographic equipment consisted of an Altex 110A high-pressure pump, a Rheodyne Model 7125 injector with a 20-μl sample loop and an Alltech 10-μm particle diameter, C_{18} chromatographic column (250 × 4.6 mm).

Detector

The POCL detector can be subdivided into two separate functional units — photochemical reactor and CL detection cell— which are connected in series via a mixing cell. All connections within the system are made with 1.5-mm Altex tube end fittings. Fig. 3 is a schematic diagram of the POCL detection system. The photooxygenation sensitizer AQDS and the luminol catalyst Co(II) are both added to the HPLC solvent reservoir, and the reservoir is oxygenated for 5 min prior to analysis. Consequently, the AQDS and the Co(II) pass through the entire HPLC system —C_{18} column, the photochemical reactor, the mixing cell and finally the CL detection cell— whereas the buffered luminol solution is introduced at the mixing cell and only flows through the CL detection cell.

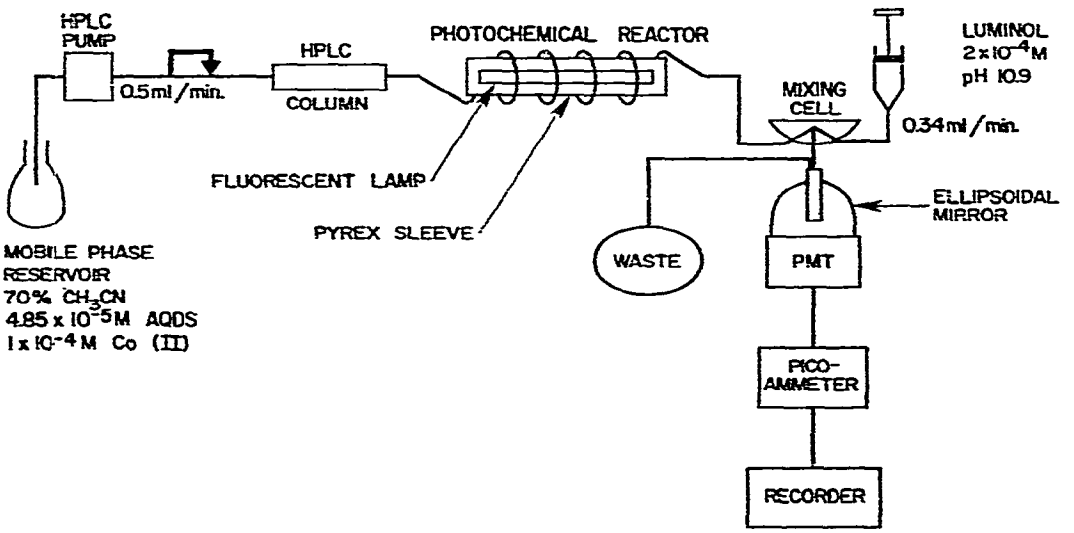


Fig. 3. Schematic diagram of the POCL detector.

The photochemical reactor cell is a 1-m length (unless otherwise noted) of 0.38 mm I.D. transparent PTFE tubing (Bel Arts Products, Pequannock, NJ, U.S.A.). Scholten *et al.*[22] have previously described the use of PTFE tubing in post-column photochemical reactors. The dead volume (220 μl) of the photochemical reactor was measured by weighing the amount of water displaced as a bubble of air transversed the length of tubing. The UV–visible absorption spectrum of the PTFE tubing used in the photochemical reactor has a transmittance of 50% at the center of the emission band of the UV source (365 nm). In order to avoid boiling the HPLC solvent, the 1-m length of tubing is coiled around a 26 × 2 cm O.D. Pyrex cylinder which slides over the fluorescent tube of the UV lamp. The fluorescent lamp (Sylvania Model E 8TS IBLB) was purchased from a local hardware store for approximately $20. Finally, the entire photochemical reactor was covered with aluminium foil to increase the photon flux by reflection.

A mixing cell constructed in our own machine shop of Kel-F (DuPont, Wilmington, DE, U.S.A.) connects the photochemical reactor with the CL detection cell. The design of the semi-circular mixing cell is described by Frei[23] and functions by causing turbulent flow at the confluence point. The inner diameters of the mixing channels are 0.38 mm, while the overall radius of the semi-circular cell is 2.0 cm. The mixing cell by a 10 cm × 0.80 mm I.D. PTFE tube. Furthermore, the mixing cell is solution (2×10^{-4} *M*, pH 10.9) which is forced into the mixing cell by an Orion Model 341A syringe pump at 0.34 ml/min. The syringe pump is connected to the mixing cell by a 10 cm × 0.80 mm I.D. PTFE tube. Furthermore, the mixing cell is placed as close as physically possible to the CL detection cell (about a 1 cm distance) in order to minimize any loss of the CL signal, since the rapid CL reaction begins at the confluence point of the mixing cell.

The CL detection cell (Fig. 4) was constructed by placing a coil of PTFE tubing —the same tubing used in the photochemical reactor— within an ellipsoidal mirror (A) (02-REM-001; Melles Griot, Danbury, CT, U.S.A.). The coil of PTFE tubing (B), which is wrapped around a 1-cm diameter metal tube (C), is positioned near the first focus of the ellipsoidal mirror, whereas the second focus of the mirror is placed on the photocathode of the photomultiplier tube (PMT) (D). Consequently, most light emitted from the PTFE coil is directly reflected to the PMT. The reflector housing and PMT are the same as described earlier for the chemiluminescent aerosol spray (CLAS) detector[11]. The metal tube which supports the PTFE coil is anchored by a 1-cm O-ring compression fitting (E), and the ends of this 1-cm diameter metal tube (F) are sealed with black RTV silicone sealer (General Electric, Waterford, NY, U.S.A.). The PTFE tubing enters and exits the ellipsoidal mirror chamber by running down the center of the metal tube. The volume of PTFE tubing exposed to the PMT is 60 μl, a value somewhat larger than the dead volume of most commercial HPLC detectors.

The emission of light from the CL reaction in the 60 μl of PTFE tubing is detected by a PMT (9658RA; EMI, Plainfield, NY, U.S.A.) contained in a thermoelectrically cooled housing (FACT 50, EMI). The PMT is wired with the anode at ground and the photocathode at −1000 V, while the output current of the PMT is measured by a picoammeter (Model 480; Keithley, Cleveland, OH, U.S.A.) and displayed on a strip chart recorder. All optimizations are based on peak height measurements and peak-to-peak measurements of noise.

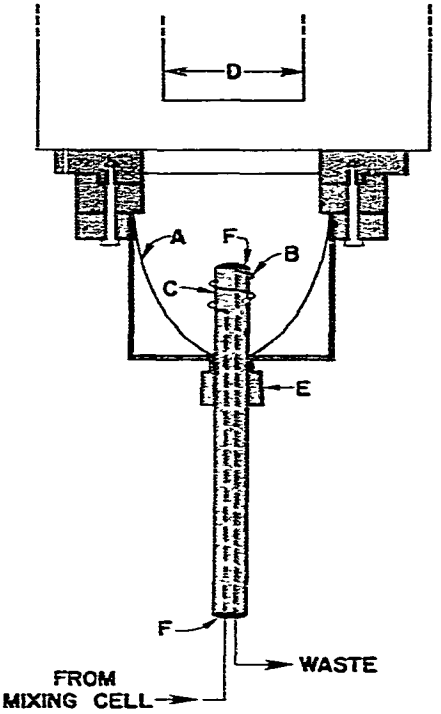


Fig. 4. Schematic diagram of the CL detection cell.

Determination of H_2O_2 chemical yield

The chemical yield of H_2O_2 in all the optimization curves except the flow-rate curve was determined by comparing the signal strengths per mole of a 20-μl injection of an H_2O_2 standard containing 7.35×10^{-8} moles with an identical injection of an isobutanol (IBA) standard containing 2.18×10^{-6} moles. For the HPLC flow-rate curve, the chemical yield of H_2O_2 was calculated using the same method; however, the H_2O_2 standard contained 7.35×10^{-9} moles, and an isopropanol (IPA) standard of 2.60×10^{-6} moles was substituted for the IBA standard. All measurements were made with the UV lamp on and the chromatographic column removed.

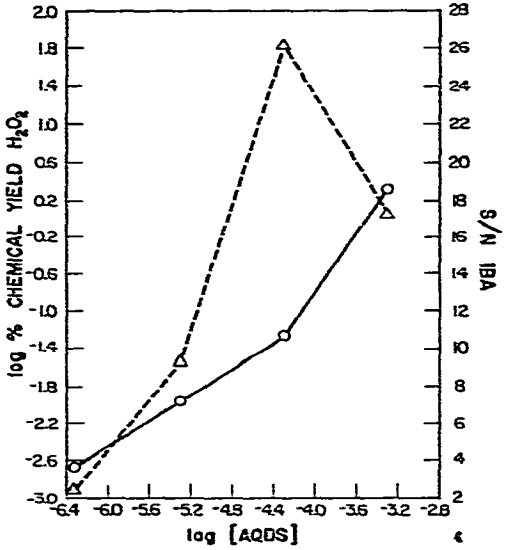
RESULTS AND DISCUSSION

Optimization of photochemistry

The sensitizer AQDS is deliberately pumped through the chromatographic column in order to improve the separation of the small aliphatic alcohols, aldehydes and ethers used in this study. Fig. 5 demonstrates the variation of the CL signal-to-noise (S/N) ratio of IBA and the chemical yield of H_2O_2 as a function of sensitizer concentration, [AQDS]. The chemical yield of H_2O_2 continues to rise with increasing sensitizer concentration, but the S/N ratio falls off after $4.85 \cdot 10^{-5}$ *M* AQDS. Consequently, $4.85 \cdot 10^{-5}$ *M* AQDS was chosen as the optimum sensitizer concentration.

The optimization of the length of PTFE tubing coiled around the Pyrex sleeve in the photochemical reactor is shown in Fig. 6. Initially an increase in the PTFE

tubing length causes a significant improvement in the S/N ratio of IBA which reaches a maximum near 1 m. As the tube length is increased beyond 1 m, the noise level rises faster than the signal level and a decrease in signal-to-noise ratio is observed. In contrast, the chemical yield of H_2O_2 continues to increase steadily as the coil length increases. Part of the decrease in the S/N ratio as the tube length increases is caused by peak broadening within the PTFE tubing coils of the photochemical reactor.



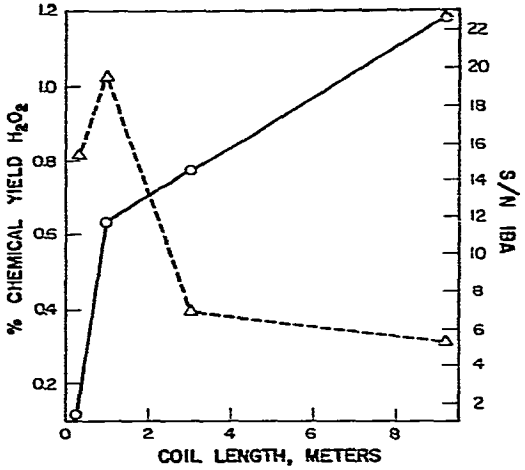


Fig. 5. Variation of signal-to-noise ratio from IBA (Δ) and % chemical yield of H_2O_2 from IBA (○) as a function of sensitizer (AQDS) concentration. Conditions: 70% acetonitrile mobile phase at 1 ml/min; 10^{-4} *M* Co(II); 3-m photochemical reactor.

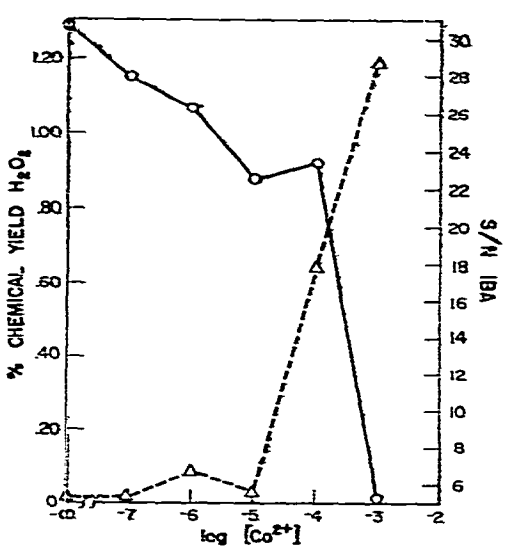
Fig. 6. Effect of photochemical reactor tubing length on signal-to-noise ratio of IBA (△) and chemical yield of H_2O_2 from IBA (○). Conditions: 70% acetonitrile mobile phase at 1 ml/min; 4.85×10^{-5} *M* AQDS; 10^{-4} *M* Co(II).

Optimization of CL reaction

The optimization for the experimental condition of the cobalt(II)-catalyzed luminol–hydrogen peroxide reaction is discussed by Burdo and Seitz[21]. They observed an optimum luminol concentration of $2.0 \cdot 10^{-4}$ *M* in a 0.1 *M* borate buffer at pH 10.9. We chose to use these concentrations and to explore only the effect of the catalyst concentration.

Cobalt(II), although not involved in the photochemistry, is pumped through the entire system including the photochemical reactor in order to eliminate the need for a second mixing cell and pump. It cannot be added directly to the luminol since — at its most effective concentration— it would precipitate out at pH 10.9 as $Co(OH)_2$. Fig. 7 exemplifies the effect of varying the cobalt(II) concentration on the CL signal height of IBA and the chemical yield of H_2O_2. Although the chemical yield of H_2O_2 decreases significantly as the concentration of Co(II) is increased from 10^{-7} to 10^{-3} *M*, the S/N ratio greatly increases for the same change in cobalt(II) concentration. Even though 10^{-3} *M* Co(II) gave the best S/N ratio, precipitation of $Co(OH)_2$ within

the mixing cell and detector cell resulted in irreproducible results. Therefore we decreased the cobalt(II) concentration to 10^{-4} *M* for further studies. At this concentration, the Co(II) causes a 30% reduction in chemical yield of H_2O_2. Thus, the S/N ratio could be somewhat improved by adding the Co(II) after the photochemical reactor. However, we chose to make this small trade-off in sensitivity for the advantages afforded by the use of one less reagent pump and mixing cell.



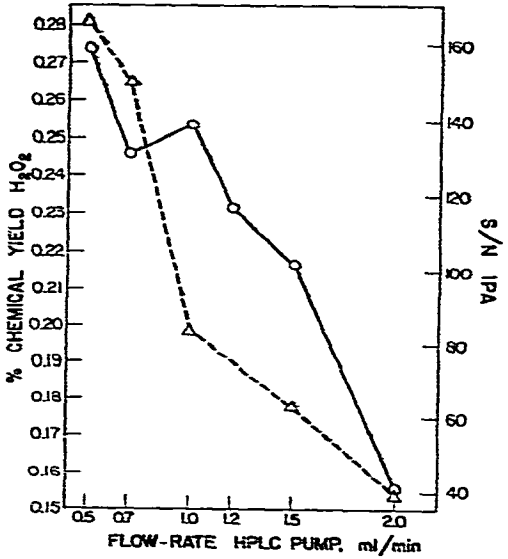


Fig. 7. Signal-to-noise ratio (△) from IBA and % chemical yield of H_2O_2 from IBA (○) *vs.* cobalt(II) concentration. Conditions: 70% acetonitrile mobile phase at 1 ml/min; 2.43 × 10^{-5} *M* AQDS; 3-m photochemical reactor.

Fig. 8. Flow-rate of HPLC pump *vs.* signal-to-noise ratio of IPA (△) and % chemical yield of H_2O_2 from IPA (○). Conditions: 70% acetonitrile mobile phase; 4.85 × 10^{-5} *M* AQDS; 10^{-4} *M* Co(II); 1-m photochemical reactor.

Effect of HPLC flow-rate

The flow-rate of the syringe pump containing the luminol solution was set at 0.34 ml/min. This value was chosen mainly by convenience. Fig. 8 demonstrates the variation of the S/N ratio of IPA and the chemical yield of H_2O_2 as a function of the HPLC pump flow-rate. The effect of HPLC flow-rate on the S/N ratio is due to a complex combination of factors. As the flow-rate of the HPLC pump is decreased, the following conditions are altered: the concentration of luminol increases while the concentrations of Co(II) and H_2O_2 decrease within the detection cell; the residence time of the analyte within the photochemical reactor increases; the dispersion due to flow through the reactor coil decreases; and the contact time between the mixing cell and CL detection cell increases. The data show an improvement in the S/N ratio as pumping rate of the HPLC pump is decreased from 2 ml/min to 0.5 ml/min. A large part of the improvement in S/N ratio with decreasing HPLC flow-rate is due to the increased chemical yield of H_2O_2 resulting from the increased residence time in the photochemical reactor. A lower limit exists for the flow-rates used in a convenient

HPLC analysis, since a flow-rate slower than 0.5 ml/min would require more than one half hour for a typical analysis.

In summary, the most suitable conditions determined by experimentation are as follows: sensitizer concentration 4.85×10^{-5} *M* AQDS; catalyst concentration 10^{-4} *M* Co(II); HPLC pump flow-rate 0.5 ml/min; and photochemical reactor length 1 m. These are the conditions used for the chromatography described below.

Coupling to HPLC

The only detrimental effect of coupling a post-column reaction detector to HPLC involves the problem of peak broadening caused by dispersion phenomena in the reactor, discussed in detail by Frei and Scholten[24]. In order to evaluate the band broadening of the POCL detector, we compared peak widths at half-height obtained using this detector with those produced by a commercially constructed 8-μl UV detector (Altex Model 153). Peak widths at half-height from the POCL detector were found to be approximately 4% greater than those from the UV detector.

Detection limits

Table I lists the detection limits for various compounds determined with the POCL detector while the C_{18} column was in place. The detection limits of the various analytes were determined by using eqn. 1:

$$m_L = 3\sigma_i/(S/m_i) \quad (1)$$

TABLE I

DETECTION LIMITS FOR COMPOUNDS TESTED

Compound	*Detection limit (μg)*
A. Alcohols	
Methanol	4.1
Ethanol	1.2
2-Propanol (isopropanol)	1.2
1-Butanol	2.1
2-Methyl-1-propanol (isobutanol)	2.1
1-Hexanol	1.5
1-Octanol	7.3
B. Ethers	
1,4-Diethylene dioxide (dioxane)	6.9
Diethylene oxide (tetrahydrofuran)	4.7
Diethyl ether	4.2
C. Aldehydes	
1,5-Pentanedial (glutaraldehyde)	11.4
Propanal (propionaldehyde)	2.6
3-Methylbutanal (isovaleraldehyde)	6.7
D. Saccharides	
Fructose	3.1
Sucrose	14.7

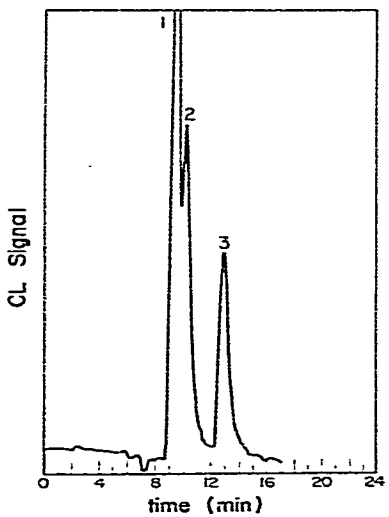
3σ corresponds to one half the peak-to-peak noise, while S is the signal obtained from an injection of a sample with a mass m_i. Unfortunately, the CL signal intensity of the analytes determined with the chromatographic column in place is not linearly related to the concentration of analyte injected; however, this problem can be circumvented by using a calibration curve.

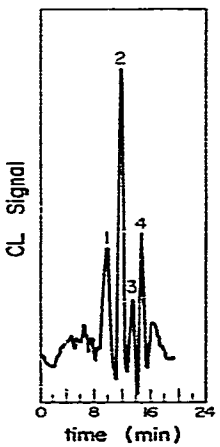
Reproducibility

The reproducibility of the POCL detector was measured with the C_{18} column in place by comparing the peak heights of ten injections of 1% IBA. The relative standard deviation was 7.80%.

Chromatography

As previously mentioned, the small aliphatic alcohols, aldehydes and ethers were difficult to separate using our C_{18} column with a mobile phase of acetonitrile–water or solely water. However, by adding the sensitizer to the mobile phase the resolution of the separations was dramatically improved. We believe the AQDS modifies the C_{18} ligands of the stationary phase by hydrophobically interacting with them, and thus the AQDS conveys a considerably more polar character to the C_{18} stationary phase. Fortunately, the addition of Co(II) to the mobile phase had no noticeable effect on the separation of the test compounds. Fig. 9, 10, and 11 demonstrate chromatograms of aliphatic alcohols, aldehydes and ethers, respectively, using the POCL detector.





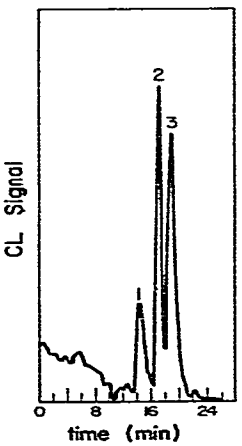


Fig. 9. Chromatogram of aliphatic alcohols. Peaks: 1 = 157 μg isopropanol; 2 = 161 μg isobutanol; 3 = 163 μg 1-hexanol.

Fig. 10. Chromatogram of aliphatic aldehydes. Peaks: 1 = 200 μg 1,5-pentanedial; 2 = 161 μg propanal; 3 = impurity; 4 = 161 μg 3-methylbutanal.

Fig. 11. Chromatogram of aliphatic ethers. Peaks: 1 = 207 μg 1,4-dioxane; 2 = 178 μg tetrahydrofuran; 3 = 141 μg diethyl ether.

CONCLUSION

The only HPLC detector presently in use and capable of detecting aliphatic oxygen compounds is the refractive index (RI) detector; however, this detector suffers from universal detection, high price and lack of sensitivity. In contrast, the POCL detector is inexpensive and selective, while having comparable sensitivity. Thus we believe that the POCL detector may offer considerable advantages over the RI detector for the determination of many oxygen containing analytes, particularly when the interpretation of complex chromatograms is required.

Two basic improvements can be made to this detection system: first, a more intense UV source should improve detection limits greatly by increasing the chemical yield of H_2O_2; and secondly, the use of a more efficient CL reagent such as TCPO might also increase the sensitivity of the POCL detector. Hopefully, with these two improvements, the detection limit may be reduced to the low nanogram range.

ACKNOWLEDGEMENT

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