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Flow injection analysis with a laser-based detector for on-line determination of sulfide, glucose, and ethanol

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FLOW INJECTION ANALYSIS WITH A LASER-BASED DETECTOR FOR ON-LINE DETERMINATION OF SULFIDE, GLUCOSE, AND ETHANOL.

By

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ABSTRACT

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In this research a flow injection (FI) apparatus with a laser-based detector is characterized using three chemical systems and the results are compared to those obtained by other researchers using conventional instrumentation. Each chemical system involves Methylene Blue (MB), a common redox indicator. A 670-nm diode laser is used as the excitation source, and the resulting fluorescence is measured. In the first system, MB is formed as a product of the reaction of N,N-dimethylphenylenediamine (DMPD), iron(III) and sulfide in the presence of acid. A three-line FI manifold is employed. The amount of sulfide in water samples, including a complicated wastewater matrix, is determined indirectly by measuring the fluorescence intensity of the MB produced in the on-line reaction. We perform the reaction under less corrosive conditions than previous researchers, and are able to make sensitive measurements (20-ppm limit of detection). For the second system under study, MB reacts with glucose, a reducing sugar, to produce the colorless leuco-MB. This system is not observed on-line due to the lack of sufficient quantities of dissolved oxygen in the flow stream. For the third system, ethanol reacts with NAD^+ in the presence of dehydrogenase, and the NADH produced can subsequently react with MB to form Leuco-MB. A decrease in MB fluorescence is observed with decreasing concentration of ethanol. Although the on-line reaction can be detected successfully, a linear relationship between the injected concentration of ethanol and signal size has not been observed yet.

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Look at the day when you are supremely satisfied at the end. It's not a day when you lounge around doing nothing; it's when you've had everything to do, and you've done it.

-- Margaret Thatcher

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Chapter 1

INTRODUCTION

The following section is designed to give an overview of the basic concepts and ideas used in this research. This section provides a basic understanding of concepts necessary in understanding flow injection analysis (FIA) and the reaction systems used in this research. In section 1, the basic ideas involved in FIA are discussed along with the instrument design. Section 2 introduces the redox indicator, methylene blue, used in all reaction systems used in this research, and sections 3, 4 and 5 introduce the three reaction systems carried out in the flow system.

SECTION 1: FLOW INJECTION ANALYSIS (FIA)

Flow injection analysis (FIA) is an automated analytical method for routine chemical, biological and environmental (1-4) analysis carried out in a flowing stream. This method is utilized for on-line titrations (5), and for a number of reactions to detect and quantitate analytes like chloride (6,7), sulfide (1), etc. It offers greater sensitivity, rapid sample rate, precise and reproducible time sequences and faster analysis in comparison to commercial routine techniques. Also, it is less expensive than conventional methods of analysis. Its low reagent consumption, small sample volume and high sample rate are desirable for routine analysis.

Principally, FIA is based on the injection of a sample into an unsegmented, continuously moving carrier stream (CS). The mixing between the injected sample and the carrier stream is always incomplete. However, due to the reproducible mixing pattern, precise results are obtained with this method. All samples follow the same mixing pattern and the detector measures the parameter of interest (absorbance, fluorescence, electrode potential, scattering, etc.) as the injected "slug" of liquid flows through the continuously moving reagent stream towards the detector.

FIA applications were first developed by Ruzicka and Hansen (6,7) in the mid-1970s when they began using an air-segmented stream (closely spaced air bubbles) in segmented flow procedures to control sample dispersion, enhance mixing of sample and reagent stream, and to prevent cross-contamination between samples. Bubbles, however, are a problem because they separate the liquid into segments which cannot mix due to the pockets of air. Mixing occurs within the segments of liquid resulting in homogeneous segments of fluid and therefore a steady-state signal is observed at the detector, which is now independent of the physical and chemical parameters of the system in use. In non-segmented systems, this problem is decreased and a higher sampling frequency is obtained. A system free of bubbles provides a higher analysis rate, faster response time and dispersion control in the system.

As a sample is injected into the carrier stream, band broadening can be observed due to dispersion of the sample zone (see Figure 1). This dispersion occurs due to convection and diffusion. Convection occurs as a result of laminar flow in which the solution in the center of the tubing moves but the fluid immediately adjacent to the walls of the tubing does not move. This increasingly slows down the rate of flow of the liquid. The shape and size of the tubing, along with the flow rate of the carrier stream, are important factors affecting dispersion. Band-broadening also occurs due to diffusion. Molecules in the faster-moving central segment of fluid move towards the slower fluid segment adjacent to the walls, and chemical species in the slower-moving segment diffuse into the faster moving central fluid segment. This results in retarding the frontal boundary of the sample zone while speeding up the rear .

Controlling dispersion is very important for the understanding of FIA. Dispersion is defined by the following equation:

$$D = C_o/C_p$$

where C_o represents the analyte concentration of the injected sample and C_p is the peak concentration at the detector (see Figure 2). Dispersion can be calculated by injecting a dye

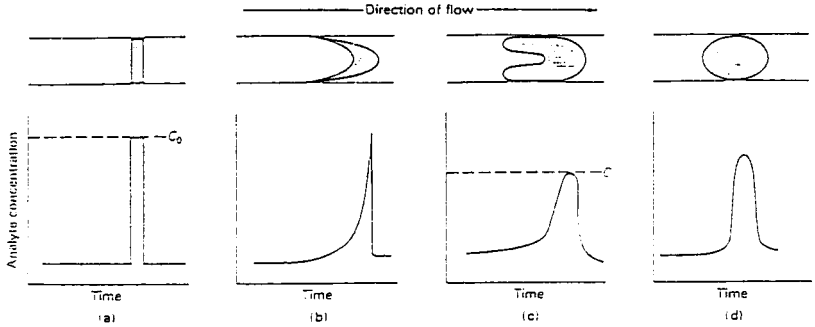


FIGURE 1: Effects of convection and diffusion on concentration of analyte at the detector. (a) No dispersion; (b) Dispersion by convection; (c) Dispersion by convection and radial diffusion; (d) dispersion by diffusion. Reprinted from (3).

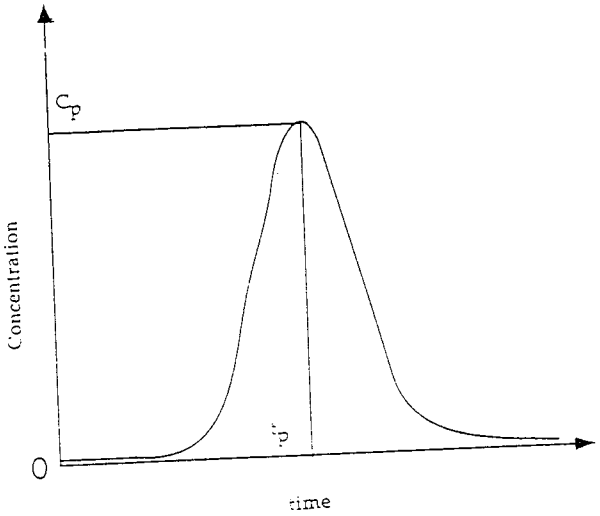


FIGURE 2: A typical FI peak. C_p is the maximum peak concentration of the analyte; t_p is the time expired after injection of the analyte. Reprinted from (3).

solution of known concentration, C_o, in a colorless carrier stream and measuring the absorbance of the dye. After calibration, C_p can be calculated by Beer's Law:

$$A = \varepsilon bc$$

where A is the absorbance, ε is the molar absorptivity, b is the path length and c is the analyte concentration.

Values of D are used to characterize the extent of mixing in the flow tubing. Generally, a D value of 1-3 is referred to as "limited" dispersion, 3-10, "medium" dispersion and greater than 10, "large" dispersion (3). Parameters like tube length, sample size and pump rate also affect dispersion. Ruzicka and Hansen reported that increasing the tube length, tube diameter, or flow rate increases the value of D, whereas increasing sample volume decreases the value of D (8). Additional methods can be used to increase diffusion and convection in the flow stream. The confluence or convergence of two carrier streams can be used to increase dispersion. Also, the addition of secondary flow patterns can increase the mixing of the sample zone and the carrier stream. Coiled, knitted or knotted tubing can further increase the tortuosity, thereby aiding in the mixing of the slower and faster moving fluid segments that result due to laminar flow (9).

The FIA apparatus in use varies depending on the reaction of interest. It could be as simple as a single line manifold (see Figure 3) consisting of a peristaltic pump (P) to regulate a continuous, pulse-free flow of the carrier stream (C or CS), an injection valve (I), a mixing or reaction coil (MC), a detector (D) and a recorder or computer. The peristaltic pump, generally designed with 8-10 rollers, squeezes the fluid through the tubing (see Figure 4). The speed of the motor, along with the diameter of the tubing used, controls the flow rate. FIA manifolds generally employ narrow-bore Teflon tubing (9) of various inner-diameters (0.5-0.8 mm). Depending on the reaction of interest, the FIA apparatus can also be complex, for instance a double- or triple-line manifold system. One of the systems in this research involved a triple-line manifold. The schematic for the

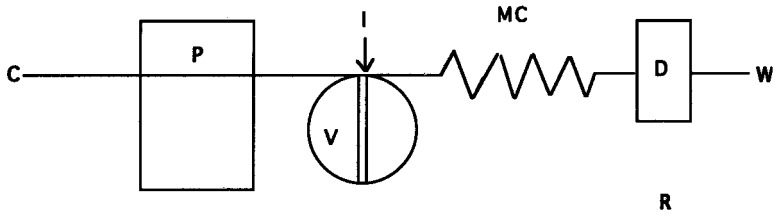


FIGURE 3: A single-line FIA manifold. C = Carrier stream, P = Peristaltic Pump, I = Injection port, V = Injection valve, MC = Mixing coil, D = Detector , R = Chart Recorder or Computer, and W = Waste.

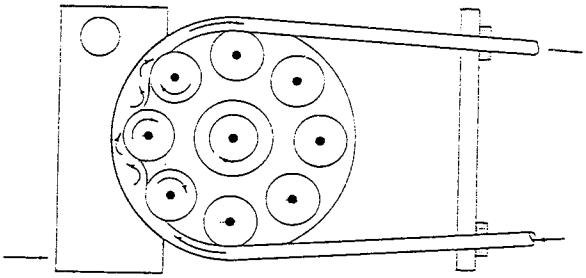


FIGURE 4: Diagram of one channel of a peristaltic pump. In a triple-line manifold, there would be three pieces of tubing placed adjacent to each other on top of the rollers. Reprinted from (3).

manifold used is shown in Figure 5. Here, two of the carrier streams flow separately and then combine in a mixing coil (MC) before converging with the third CS into which the sample is injected. All the reagents then mix in the reaction coil (RC) before being carried to the detector.

In conjunction with the FIA apparatus, a diode-laser-based detector is used for the detection of the desired analyte. Lasers and lamps are used commonly today as sources for optical radiation. Lamps provide a stable and large output power for spectroscopic measurements, but there are also problems like incoherent emission and tuning incapability (9). A laser (*L*ight *A*mplified by *S*timulated *E*mission of *R*adiation) is advantageous because it has beam focusing capability, selectivity and high photon flux (10). Diode lasers are small, inexpensive, semiconductor-based devices (11) that provide narrow single-mode linewidth, tunability, stable emission wavelength and rapid response times (12). High output power with high electrical to optical efficiency (13), low cost (14) and low noise due to lack of internal optical elements (15) make diode lasers an attractive choice as the source for optical spectroscopy. In this research, a group III-V compound semiconductor-based laser that emitted in the visible region was utilized.

The miniature detector used in this research was designed and constructed by James L. Davis, a former Union College student as part of his thesis research project (16). The circuitry for the detector was developed by Marc A. Unger (17). The instrument is compact, economical and solid-state, consisting of a diode laser with two photodiodes at 90 and 180 degrees to the laser (see Figure 6) for simultaneous fluorescence and absorbance measurements. Photodiode detectors detect intensity of light with high sensitivity. These detectors are small and inexpensive, provide rapid response and offer a wide linear range of transmitted light. Their operation is based on the photovoltaic effect where a voltage is generated across a p-n junction when the photodiode is exposed to a light source. With the use of lenses and filters, the required wavelength is isolated (17), the photon signal is converted to an electrical signal (voltage), amplified, noise removed and finally the data

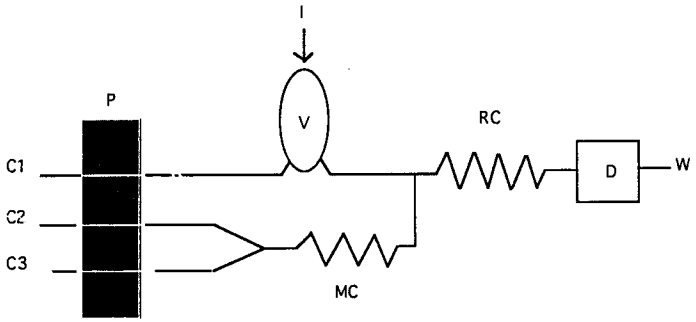


FIGURE 5: Schematic diagram of a triple-line FIA manifold. P = Peristaltic Pump, I = Injection port, V = Injection valve, MC = 200 x 0.5 mm Mixing coil, RC = 2000 x 0.5 mm reaction coil, D = Detector, W = Waste, and C = Carrier Stream. For the *N,N*-Dimethyl-*p*-phenylenediamine (DMPD) system, C1 is 25.0 mM NaOH, C2 is 16 mM $FeNH_4(SO_4)_2$ in 0.100M H_2SO_4, and C3 is 2g/L DMPD in 0.5M H_2SO_4. Here, C2 and C3 flow at the same rate; C1 flows at twice the flow rate of C2 and C3.

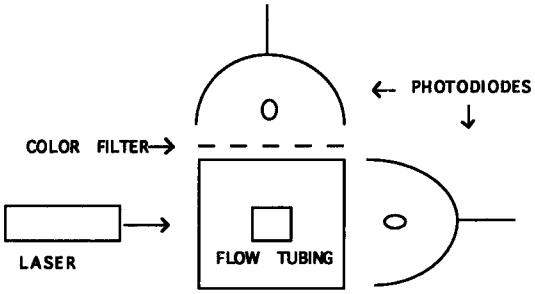


FIGURE 6: Alignment of the photodiodes with respect to the laser. The photodiodes are placed at 180 and 90 degrees to the laser for absorbance and fluorescence measurements, respectively. The capillary flow tubing carries the reagents (flow direction is in and out of the paper).

transported to the computer.

SECTION 2: METHYLENE BLUE (MB)

For the current research, Methylene Blue (MB), a basic, organic, blue dye was used. MB absorbs strongly around 670-nm and subsequently fluoresces at higher wavelengths. In this research, a 670-nm diode laser was used. MB is highly soluble in water but, like other polymethine dyes, it photodegrades in an aqueous solution. At $pH \geq 1$, the acidic form of MB, MB^+, exists in solution and the characteristic peak absorbance of this species is seen at 664-nm. However, with increasing acidity, a red-shift in the absorbance spectrum is seen with a maximum absorbance at a wavelength of 745 nm representing the MBH^{2+} form of the dye. For our experimental setup with the 670 nm laser as the excitation source, MB was prepared in acid of $pH \geq 1$.

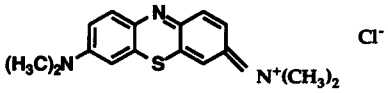
MB is commonly used as a detector for redox reactions (14,18,19) because a reversible redox reaction can take place whereby MB is reduced to its colorless form, leuco-Methylene Blue (see Figure 7 for structures of MB and Leuco-MB).

$$\text{MB (blue)} + 2H^+ + 2e^- \Leftrightarrow \text{Leuco-MB (colorless)}$$

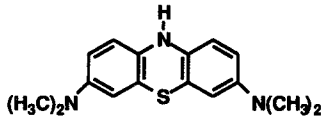
Leuco-MB converts back to the blue form easily in the presence of an oxidizing agent. This reaction has been used to measure electrode potential and enzyme activity, and also for staining DNA (10), elastic fibers, animal tissue and connective tissue (20). In this research, MB is used for determination of sulfide (1), glucose (19) and ethanol (18).

SECTION 3: DMPD SYSTEM

This system is a well-known method for sulfide analysis (1,2,19) and was first reported by Fisher et al. in 1883 (21). It involves the production of MB upon the oxidative



Methylene Blue Chloride



Leuco-Methylene Blue

FIGURE 7: Chemical structures of MB and Leuco-MB.

coupling of sulfide with *N,N*-dimethyl-*p*-phenylenediamine (DMPD), or DMPD in salt form, in the presence of iron (III). The mechanism for this reaction is not very well understood. Boltz and Howell (20) suggested the mechanism shown in Figure 8. However, a more elaborate, six-electron exchange reaction was recently proposed by Kuban et al. (see Figure 9). Kuban and his group (1) used this method for sulfide analysis in a flow-injection system. The goal of our research was to reproduce the results obtained by Kuban et al. for the characterization of the new instrumentation (16,17). In her senior thesis, Michelle Spaziani (9) presented these reproduced results with higher detection limits and sensitivity than Kuban and his group. This research is a continuation of Spaziani's work, and some of her results are reproduced here.

In the research presented by Kuban et al., absorbance measurements were made for sulfide analysis. However, a fluorescence-based detector can be more sensitive than an absorbance-based one, and therefore we measured MB fluorescence. With this increased sensitivity due to fluorescence detection with a diode-laser-based detector, we could perform the reaction on-line under less corrosive conditions than those reported in the literature (1).

SECTION 4: GLUCOSE SYSTEM

Methylene Blue has been reported as an indicator for a variety of redox reactions (14, 18, 19). Fenster and coworkers (19) presented a demonstration to show the reaction of MB to colorless leuco-MB in the presence of a reducing agent. If MB is added to glucose in a basic solution, the resulting solution is colorless. However, if the solution is stirred to introduce atmospheric oxygen into the solution, the leuco-MB is oxidized back to MB, resulting in a blue solution. Our goal was to perform this reaction on-line in a flow injection system. Originally, the idea was to use a single-line manifold (as shown in Figure 10) where the MB solution would provide a steady state fluorescence signal until glucose

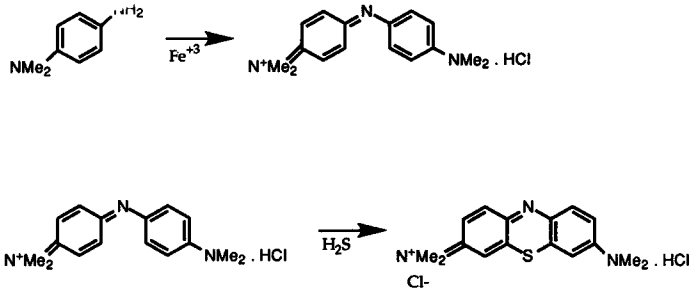
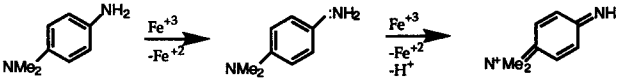
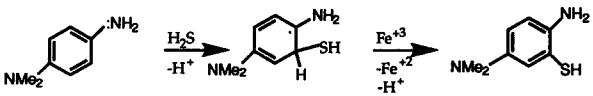


FIGURE 8: "MB Method" Reaction mechanism as proposed by Boltz and Howell (20).

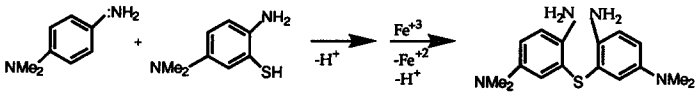
a) Formation of a cation radical and quinonediimine.



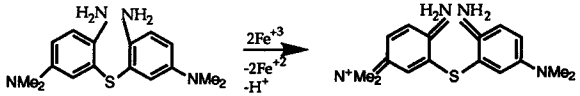
b) H_2S trapping of a cation radical.



c) Cation radical initiated dimerization.



d) Reoxidation to a quinonediimine and 1,2 addition.



e) Elimination of ammonia to form methylene blue.

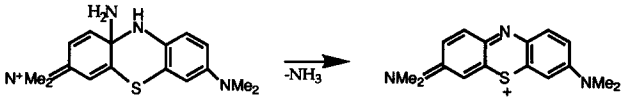


FIGURE 9: "MB Method" Reaction mechanism as proposed by Kuban et al. (1)

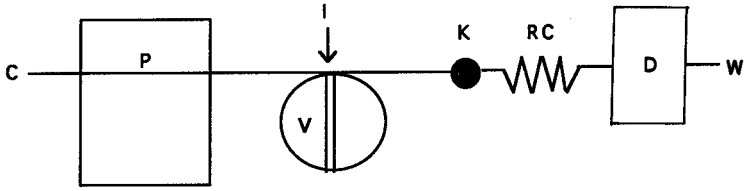


FIGURE 10: A schematic of a single line FIA manifold used for the glucose system where C = Carrier stream, P = Peristaltic Pump, I = Injection port, V = Injection valve, MC = Mixing coil, K = Knotted flow tubing, RC = Reaction coil, D = Detector (Computer), and W = Waste. The carrier stream is glucose in base and MB is injected at I; alternatively, the carrier stream contains MB and glucose in base is injected at I.

was introduced in a basic solution, and due to the production of leuco-MB, a decrease in fluorescence would be seen. Due to several problems however, experiments with glucose in base as the carrier stream were also conducted. Here, MB was injected into the glucose carrier stream. In this set-up, decreasing fluorescence intensity of MB due to the formation of leuco-MB should be observed with larger allowed reaction time. As of now, this system has not worked on-line due to problems with dispersion and noise, longer required reaction time, and low rate of oxygen diffusion into the carrier stream through the flow tubing for the oxidation of leuco-MB to MB.

SECTION 5: ETHANOL SYSTEM

This reaction system utilizes MB as an indicator in a redox enzyme assay which can be used for analysis of blood alcohol in the body. Imasaka and coworkers (18) have worked on this reaction where ethanol, in the presence of NAD^+ (nicotinamide adenine dinucleotide) and alcohol dehydrogenase (ADH) enzyme, forms acetaldehyde as the product along with NADH in acid.

$$\text{ethanol} + NAD^+ + \text{ADH} \Rightarrow \text{acetaldehyde} + \text{NADH} + H^+$$

Bradley et al. (22) worked on the same reaction and came up with a Centrifugal Analyzer for the quantitation of ethanol, based on the measurement of NADH fluorescence. Imasaka, however, used an indirect method for the detection of ethanol. The amount of NADH produced can be detected by its reaction with MB; NADH reduces MB to the colorless leuco-MB in the presence of diaphorase in acidic solution.

$$\text{MB} + \text{NADH} + \text{diaphorase} + H^+ \Rightarrow \text{Leuco-MB} + NAD^+$$

Our goal is to carry out this indirect method for ethanol analysis on-line where ethanol can be injected into a triple-line manifold containing all the chemical species, and the reaction can be detected by the decrease in fluorescence of MB upon the formation of leuco-MB.

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Chapter 2

EXPERIMENTAL

This chapter presents the experimental materials and procedures for the setup and maintenance of the instrument, and the procedures for the experiments conducted with each reaction system.

SECTION 1: MATERIALS AND PROCEDURES FOR INSTRUMENT SET-UP

The triple-line FIA manifold used for the DMPD reaction is presented in Figure 5. The Tygon pump tubing of 0.64 mm and 0.44 mm inner diameter (i.d) was purchased from Cole Palmer and Teflon flow tubing of 0.5 mm i.d. was purchased from Upchurch Scientific. A variable-speed, four-channel peristaltic pump from Ismatic-sa and a six-port sample introduction valve was purchased for the set-up. Spaziani (1) calculated the injection loop volume to be 69.6 ± 0.9 μL. Flangeless fittings like Tefzel nuts and ferrules, and long FIA coilers from Upchurch were used in conjunction with three-way connectors from Cole Palmer for the manifold. The 670-nm diode-laser (Power Technology) and PIN photodiode detectors (Honeywell) were used with a Roscolux color filter (Medium Blue #83) to reduce scattering in the fluorescence measurements. Figure 6 shows the set-up of the photodiode detectors with respect to the laser and the quartz capillary tubing (Wale Apparatus) that was used to carry the solution through the mount for the laser and the photodiode detector.

SECTION 2: DMPD SYSTEM

MB was purchased from Eastman Kodak (Rochester, NY), and Aldrich (Milwaukee, WI), sulfuric acid (18.0 M) and sodium hydroxide from Fisher (Fairlawn, NJ), DMPD and sodium sulfide ($Na_2S.9H_2O$, 98%) from Aldrich and ferric ammonium sulfate from Merck (Rahway, NJ) (1).

The method used for sulfide analysis was similar to that used by Kuban and group (2), except for the acidity of the DMPD solution. Kuban et al. reported results with 9 M sulfuric acid or 6M hydrochloric acid (2). Spaziani compared results to Kuban et al. with decreasing acidity (9, 4.5, 2, 0.5 M Sulfuric acid) of the DMPD solution (1). In this work, 2.00 and 0.100 M sulfuric acid was used. DMPD solutions were prepared by the addition of 500 mg of the salt to 250 ml of the corresponding acid. Ferric ammonium sulfate solutions were prepared in 0.100 M sulfuric acid. Stock sulfide solutions were prepared daily in aqueous sodium hydroxide (made with boiled DI water).

SECTION 3: GLUCOSE SYSTEM

MB and α-D-glucose were purchased from Aldrich, potassium hydroxide and sulfuric acid from Fisher, and absolute ethanol from Quantum Chemicals. For the demonstration in the literature (3), large quantities of glucose and base were used. To avoid problems of corrosion of tubing from a strongly basic solution, the concentration of base for the glucose solution was chosen to be approximately 0.1 M. MB was prepared in 0.05 M sulfuric acid to avoid the photodegradation of MB that occurs in an aqueous solution. We found that at a pH below 12.5, the reaction in a beaker cannot be observed visually. Therefore the pH of the solution was designed to be 12.5 .

To increase dispersion and thereby increase the extent of reaction, a knot was made out of the Teflon flow tubing. To further increase the extent of reaction between the carrier stream and the injected sample, the peristaltic pump was shut off 70 s after sample injection for 0, 2 or 4 minutes, the allowed reaction time. It took 70 s for the whole injected slug of liquid to reach the knot when a pump speed of 10 rpm was employed. Fluorescence intensity for uninterrupted runs could then be compared with runs where the pump was stopped for varying lengths of time to increase the duration of the reaction.

SECTION 4: ETHANOL SYSTEM

For this system, MB was purchased from Aldrich, absolute ethanol from Quantum Chemicals, diaphorase enzyme (DP) and mono-sodium salt of NAD^+ from Amersham and alcohol dehydrogenase (ADH) from yeast from the Sigma Chemical Company.

Because the reaction could not be detected on line at first, it was tried in cuvettes with conventional instrumentation. Here, all components of the reaction mixture were added in the cuvette except NAD^+ (system A) or ethanol (system B). Originally the concentrations used were from the literature (4). However, when the reaction could not be detected on-line, ten times the reported concentrations were used. For these studies, 50 nmol of NAD+ (prepared by adding 72 mg of the mono sodium salt of NAD^+ to 10 ml DI water), 1 Unit/ml of DP (prepared by adding 26.7 mg of DP to 10 ml DI water), 6.0 Unit/ml of ADH (prepared by adding 26.7 mg of ADH to 10 ml DI water, Tris Buffer (prepared by adding 5.0 ml of 1.0 M tris(hydroxymethyl) aminomethane solution and 10.3 ml of 0.1 M HCl in water to give a total volume of 100 ml), and 5.00×10^{-6} M MB (prepared by adding 0.187 g MB to 50 ml of 0.001 M HCl to get 5.00×10^{-3} M MB and performing serial dilutions to give the required concentration in DI water). For system A, 0.5 ml each of absolute ethanol, DP, ADH, MB solutions and 1.00 ml of the buffer solution were added. For system B, a similar solution was prepared; instead of ethanol however, 0.5 ml of NAD^+ solution was added.

Measurements for this study were carried out on the Perkin-Elmer LS-5B Luminescence Spectrophotometer, equipped with 4 cuvette holders. For all the experiments conducted, quartz cuvettes were used, because signals collected for solution in the disposable polyethylene cuvettes were very noisy. Fluorescence was measured before, immediately after, and at five-minute intervals for an hour after addition of NAD^+. Similarly, for system B, all components of the reaction mixture were added in the same proportions, and fluorescence measurements were taken before, immediately after and at

five-minute intervals for an hour after ethanol addition. Also, both systems A and B were carried out in cuvettes where the solution was being constantly stirred and was open to the atmosphere, and in capped cuvettes stored in the dark. Since the reaction came to a rapid equilibrium, seen as the quick leveling off of the measured fluorescence intensity, when the cuvettes were left open and were stirred between measurements, reproducibility studies were not carried out for this experiment. When the cuvettes were capped, the decrease in MB fluorescence intensity was clearly observed. The initial decrease in MB fluorescence was markedly larger for system A, where NAD^+ was added to the reaction mixture, in comparison to system B, where ethanol was added to the mixture. This was also observed by Imasaka and coworkers (4). Therefore, the reproducibility studies were carried out only on system A.

Next, the reaction was tried on-line in a single-line manifold. Similar to the studies carried out in cuvettes, the carrier stream for the flow system was prepared by combining 0.5 ml each of DP, ADH and MB solutions, 1.00 ml of the buffer solution, and 0.5 ml of NAD^+ or absolute ethanol, for systems B and A, respectively. This solution was the carrier stream and ethanol or NAD^+ was injected into the system as the "analyte". To check whether each reaction was in fact taking place in the flow system, and that the observed signal was not due to dilution of the MB in the carrier, water and ethanol were also injected into the carrier stream.

Since ethanol and water injections in the flow system resulted in different responses for MB fluorescence, a short study was also carried out to see if MB absorbance values in ethanol and water were significantly different. This study was conducted with a conventional spectrophotometer (Perkin-Elmer LS-5B Luminescence Spectrophotometer).

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Chapter 3

RESULTS AND DISCUSSION

SECTION 1: DMPD SYSTEM

Spaziani (1) reproduced the results presented by Kuban et al. (2) for the characterization of the instrumentation, with higher limits of detection (LOD) than those presented in the literature. Spaziani (1) also found that the results could be obtained with higher sensitivity under less corrosive conditions in comparison to literature values. Therefore, in this research only 2.00 and 0.100 M sulfuric acid was used for the DMPD solution in comparison to 9.0 M acid used by Kuban and his coworkers (2).

Spaziani (1) reported "dips" in fluorescence intensity as MB formed as the product at the detector. The reported data was manipulated to show peaks instead of dips and was corrected for the background signal. Similarly, the data collected for this research was also seen as dips, but it was not manipulated to represent peaks. The data was, however, corrected for the background signal.

All the data collected is based on the average of three to four replicate sulfide injections of varying concentrations. With both 2.00 and 0.100 M DMPD solutions, calibration curves were made with sulfide concentrations ranging from 6.30 to 90.0 mg/L (2.62×10^{-5} to 3.74×10^{-4} M) (see Figures 11 and 12). Both calibration curves were found to be linear over the range of concentrations used, with r^2 values of 0.989 and 0.995 for 2.00 M and 0.100 M sulfuric acid in the DMPD CS, respectively. Thus, the fluorescence signal seen from the production of MB on-line is linear with respect to the injected sulfide. Further, increased sensitivity (lower LOD) is seen with the DMPD solution prepared in 0.100 M sulfuric acid solution, the lowest concentration of acid used, in comparison to higher concentrations of acid.

Unlike the clear, colorless DMPD solutions prepared with high acid concentration, the 0.100 M DMPD solution is purple; its color is observed to intensify over time. The fluorescence intensity of MB in 2.0 M and 0.100 M acid similar. However, the signals in response to the on-line production of MB are smaller (1). The absorbance of the purple

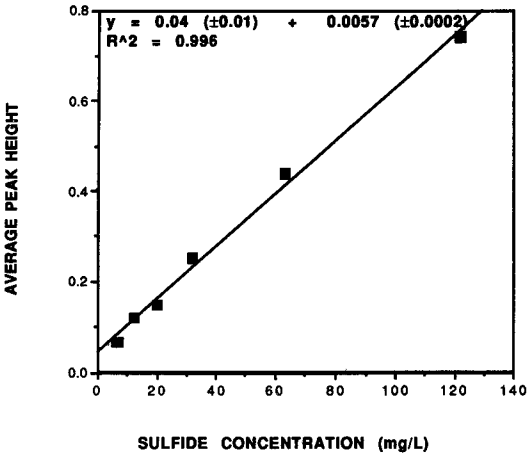


FIGURE 11: Fluorescence response to sulfide injections of varying concentrations. DMPD carrier stream prepared in 0.100 M acid.

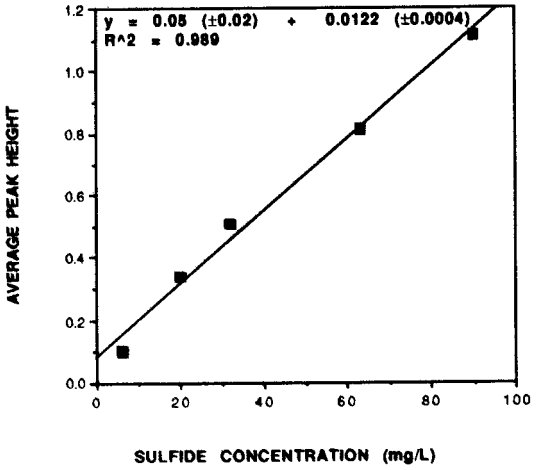


FIGURE 12: Fluorescence response to sulfide injections of varying concentrations in DMPD carrier stream prepared in 2.00 M acid.

0.100 M solution is, of course, higher than the colorless solution of DMPD in 2.00 M acid (1). Spaziani hypothesized the presence of an intermediate accounting for the purple color, which would also decrease the amount of DMPD available for reaction, thus giving smaller signals. Yet, the system would still function as before, and therefore a linear response to increasing sulfide concentration is observed. We, like Spaziani, report our best limit of detection with the 0.100 M acid, which is the least acidic solution employed. Thus, comparable results to literature values (2) are seen under less corrosive conditions (0.100 M vs. 9.0 M sulfuric acid used in literature (2)).

Unknowns used were sulfide solutions of known concentration, and sulfide stock solutions of known concentration in a simulated waste-water matrix. This waste-water matrix was the same mixture used by Kuban and coworkers (2) and was designed to test the effects of impurities that might be present in a realistic waste-water sample on the sensitivity of the system. The waste water matrix contained phenol, CH_3COONa, NaCl, KCl, $CaCl_2$, KSCN, Na_2CO_3, $Na_2S_2O_3$ and $(NH_4)_2SO_4$, compounds that may be present in waste-water samples (2). Table 1 presents the calculated concentrations of unknown sulfide samples used with the calculated errors associated with the measurements. (Spaziani's results are also presented for comparison of error in calculated concentrations.) The errors can be attributed to injection noise, and due to varying levels of environmental noise. The latter might arise from taking data on different days. To reduce this effect, all the data for each calibration curve was taken on the same day. As seen from Table 1, the response to sulfide in the waste water matrix was not much different from sulfide determined alone in the system. In both cases, the signals were reproducible (Figure 13, a and b) and comparable in height (Table 2). Again, an average of three to four replicate sulfide injections was used and no effort was made to smooth the data. A Linear Least Squares Program on Excel (Microsoft) was used for analysis of the unknowns and for the calculation of the background and LODs (3 σ above background). The results obtained by the analysis of sulfide unknowns in the system and in the waste water matrix suggest that

TABLE 1: Injected and determined concentrations of sulfide. Differences between the calculated concentrations of just sulfide vs. sulfide prepared in the waste water matrix can also be seen. The errors in calculated sulfide concentrations can be compared with Spaziani's results [Reprinted from (1)].

Conc. of acid in DMPD CS (M)	Actual conc. of injected sulfide (M) ($\times 10^{-5}$)	Determined conc. of injected sulfide (M) ($\times 10^{-5}$)
0.100M (In NaOH)	21.3	19.5 (±0.8)
	9.82	9.3 (±0.2)
(In Waste Water)	21.3	20.8 (±0. 7)
	9.82	8.0 (±0.2)
2.00M (In NaOH)	19.0	22.7 (±0.8)
	9.49	9.9 (±0.2)
(In Waste Water)	19.0	19.4 (±0.1)
	9.49	10.2 (±0.2)
	SPAZIANI'S RESULTS	
(In Waste Water)		
0.100	51.6	30 (±3)
	12.9	7 (±3)
	5.16	5 (±3)
2.00	51.6	41 (±2)
	12.9	12 (±1)
	5.16	5 (±1)
	2.58	4 (±2)

TABLE 2: Comparison of average peak heights for sulfide detection in the DMPD system (with 0.100 M or 2.00 M acid) measured for sulfide prepared in base vs. sulfide prepared in the waste water matrix. Peak heights are reported with a Gain of 10^6.

Conc. of injected Sulfide (mg/L)	23.6	51.3
Avg. Height of peak in soln. of S^{-2} prepared in NaOH, **0.100 M** DMPD (mV)	0.177	0.319
Avg. Height of peak in soln. of S prepared in waste water matrix (mV)	0.158	0.337
Conc. of injected Sulfide (mg/L)	22.8	45.6
Avg. Height of peak in soln. of S prepared in NaOH, **2.00 M** DMPD (mV)	0.360	0.719
Avg. Height of peak in soln. of S prepared in waste water matrix (mV)	0.368	0.626

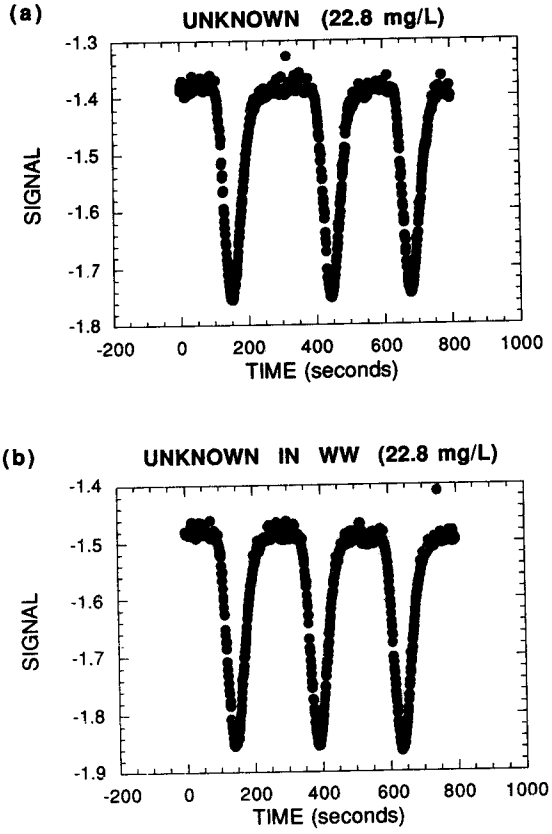


FIGURE 13: Reproducible signals as fluorescence response to sulfide injections of 22.8 mg/L.. Signals are for sulfide prepared in (a) NaOH and (b) waste water matrix.

unknown sulfide samples can be analyzed with this system with high precision.

The limits of detection presented here can be compared with values reported by Kuban et al. (2) and by Spaziani (1). The LODs presented by Kuban's group ($1\mu g/L\ S^{-2}$, or $3.1 \times 10^{-8}\ M\ S^{-2}$) are ambiguous, as stated by Spaziani, because it is not reported whether the LODs are for the concentration of injected sulfide, or the concentration of MB at the detector. If the LOD is based on the concentration of MB at the detector, then Spaziani's results (LOD = 2.4×10^{-6} M in 9.0 M sulfuric acid) are comparable to those of Kuban and coworkers. Our LOD for sulfide with 0.100 M acid in the DMPD CS was calculated to be 11 ± 2 mg/L ($4.6 \times 10^{-5} \pm 8 \times 10^{-6}\ M\ S^{-2}$). With 2.00 M acid in the DMPD CS, the LOD was found to be 12 ± 3 mg/L sulfide ($5 \times 10^{-5} \pm 1 \times 10^{-5}\ M\ S^{-2}$).

SECTION 2: GLUCOSE SYSTEM

On-line detection of glucose in the FIA system used in this research has not been accomplished. As mentioned earlier, two different methods were tried, one with glucose in the CS and the second with MB in the CS. Due to several problems, the reaction has not even been detected to take place on-line. However, in the process of trouble-shooting, several problems seen earlier have been eradicated.

At times during the work on the DMPD system, a lot of noise and baseline shifts were observed at detector gain settings of 10^6 and 10^7. The noise signal was seen recurring at approximately 650 ms (1.5 Hz) intervals. The source of the noise was found to be the circuit box used for the photodiode. Technically, the circuit box should be grounded to the wall by the plugs used. However, we found that once the box was externally grounded, the noise seen at 1.5 Hz was eliminated.

Also, as Spaziani (1) had seen, we were also measuring dips instead of peaks for MB production on-line in the DMPD system. In the process of trouble-shooting the

glucose system, it was discovered that the interference filter used between the sample stream and the photodiode to reduce scattering was misaligned. Once the filter was aligned correctly, however, a rise in fluorescence due to the detection of MB was observed as peaks. This meant that laser scatter reaching the detector along with the signal had caused the formation of dips instead of peak signals during data collection. The data collected for the DMPD system is still reliable and reproducible because all the data was collected in the same manner, and under the same conditions. The production of MB on-line in the DMPD system would still be proportional to the fluorescence intensity measured. Now, an increase in fluorescence intensity is observed as a peak instead of a dip.

To increase the mixing of the reagents for the glucose reaction, we tried to increase the dispersion of the system. A knot with the flow tubing was made and was placed before the mixing coil to enhance dispersion in the system. During measurements, the peristaltic pump was shut off for varying amounts of time after the slug of injected MB or glucose reached the knot. Even with 2 or 4 minutes of allowed reaction time in the knot, the reaction between glucose in base and MB was not detected. A slight decrease in fluorescence of MB was seen with increased reaction time. However, this result was not reproducible. Perhaps a longer reaction time is required for this reaction to take place on-line. Another factor to look into is a further increase in dispersion.

Another problem encountered in this work was the staining of the tubing with MB. If glucose is used as the CS, and the MB injected is prepared in water, the MB peak does not return back to the baseline and stains the tubing. If the MB is prepared in 0.1 M H_2SO_4, the tubing is not visibly stained; yet, after a peak, the signal does not return all the way back to the baseline. Absolute ethanol was used as a wash to remove the MB that was bound to the tubing, so that the baseline does not change due to an accumulation of MB in the flow tubing. After the interference filter was changed and realigned, the MB fluorescence peaks from ethanol washes were smaller. However, peaks observed from ethanol injections into a glucose CS suggest a difference in refractive index, resulting in a

signal. Also, MB in ethanol was found to have a higher molar absorptivity than in water or acid, because the absorption spectrum of MB in ethanol gave a higher absorbance value at 656 nm than MB in water or acid (see page 43). Other solvents that could be used for washes to remove MB need to be investigated.

The glucose solution prepared in potassium hydroxide was observed to turn from clear and colorless to clear yellow after one day. This yellow color stays regardless of how the solution is stored, in an air tight volumetric or in an open container. It was also observed that the color change only took place in the solution that was kept in an air-tight volumetric flask. The same colorless solution kept in a beaker, left open to the atmosphere, did not undergo the color change. However, the color change resulting in a yellow solution did not seem to change the reactivity of the glucose solution when the reaction was carried out in the beaker.

In the process of trying to make this reaction work in a flow-injection system, a lot of problems were solved. The noise at 1.5 Hz was eradicated and scattering was reduced by replacing and realigning the interference filter. However, as of now, glucose cannot be detected by performing this reaction on-line. This is probably due to a longer reaction time required for this reaction to take place in a FIA system and a higher dispersion needed for better mixing of the reagents in the flow tubing. The Teflon® flow tubing used in the experimental setup is air-permeable, however, rapid oxidation of leuco-MB is not observed in the flow system due to a low rate of oxygen diffusion through this tubing. Thus, due to a longer reaction time, lack of oxygen in the carrier stream and dispersion problems, this reaction has not been performed successfully on-line.

SECTION 3: ETHANOL SYSTEM

The goal of this research was to reproduce results of Imasaka's group (3) on line in the FI system. We measure indirectly the amount of NAD^+ or ethanol added, by measuring the decrease in fluorescence intensity of MB.

$$\text{ethanol} + NAD^+ + ADH \Rightarrow \text{acetaldehyde} + NADH + H^+$$

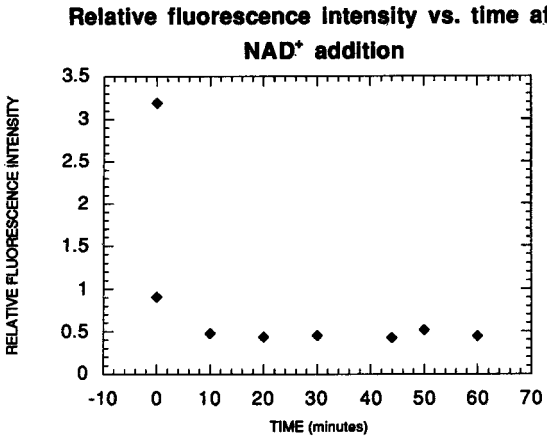
$$MB + NADH + \text{Diaphorase} + H^+ \Rightarrow \text{Leuco-MB} + NAD^+$$

With the concentrations reported in the literature (3), the reaction could not be detected on line; thus, a series of preliminary studies were carried out with ten times the reported concentrations. These studies were carried out with conventional instrumentation, followed by experiments on line.

Preliminary experiments with conventional instrumentation

For the studies carried out with conventional instrumentation, four experimental conditions were tested: system A (NAD^+ added to solution with all other components) and system B (ethanol added to solution with all other components) with closed, capped cuvettes and open cuvettes in which the solution was being stirred constantly.

When the cuvettes were open to the atmosphere and were stirred between measurements, an equilibrium was observed for the fluorescence intensity of MB in both systems A and B. Figure 14 shows the relative fluorescence intensity vs time curve observed when system A and B reactions were carried out under these conditions. The rapid equilibrium observed is due to the availability and diffusion of atmospheric oxygen, which is responsible for oxidizing the leuco-MB back to MB in the reaction system.



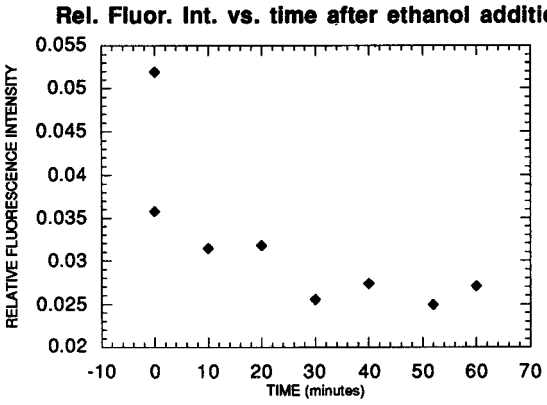
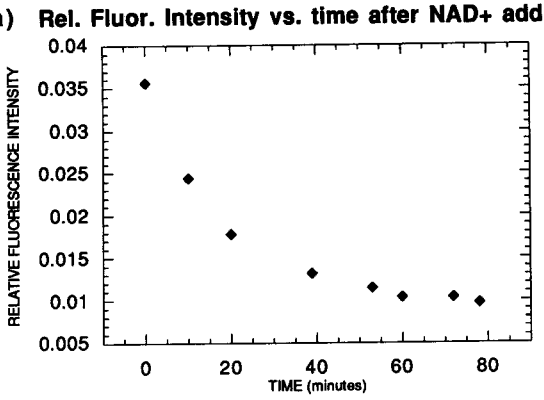


Figure 14: Relative Fluorescence Intensity vs. time after addition of NAD^+ and absolute ethanol in open cuvettes that were stirred between measurements.

$$MB\ (blue) + 2H^+ + 2e^- \Leftrightarrow Leuco\text{-}MB\ (colorless)$$

This oxidation of leuco-MB to MB by oxygen is not desirable since it prevents measurement of the decrease in MB fluorescence intensity due to the reaction of MB with NADH. In the flow system, the rate of diffusion of oxygen through the flow tubing is slow, and so the reaction of MB with oxygen is not anticipated to be a major problem. Therefore, further studies involving stirred solutions in open cuvettes were not carried out. However, it is important to note that the initial drop in MB fluorescence for this reaction condition is much greater for system A where NAD^+ is added to the reaction mixture, than for system B where ethanol is added to the reaction mixture (see Figure 14). This result was also observed by Imasaka and coworkers (3). However, it is not clear why this occurs.

Next, system A and B reactions were carried out in capped cuvettes that were stored in the dark throughout the experiment. Figure 15 shows the relative fluorescence intensity vs time curves observed when system A and B reactions were carried out under these conditions. Smoother decay curves are seen under this experimental condition than if the solutions are stirred in cuvettes open to the atmosphere. These smoother curves are due to the relative lack of oxygen which acts as an interferent in the reaction system. Thus, the better experimental condition for conducting reproducibility studies is with solutions in capped cuvettes that are stored in the dark. As seen with the open stirred cuvettes, for system B (ethanol added to the reaction mixture), the initial fluorescence signal observed before the addition of ethanol was much smaller than the signal observed for system A (NAD^+ added to the reaction mixture). Consequently, the change in signal seen after ethanol was added was also very small. Therefore, the reproducibility studies were conducted solely with system A, where NAD^+ was added to the reaction mixture, because the initial decrease in MB fluorescence intensity in this case is much greater in comparison to a system where ethanol is added to the reaction mixture.



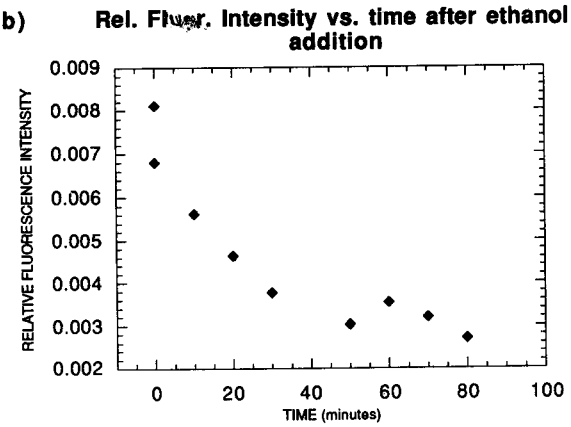


Figure 15: Relative Fluorescence Intensity vs. time after addition of NAD^+ and absolute ethanol in closed cuvettes that were stored in the dark. In a) the initial MB fluorescence intensity before addition of NAD^+ was approximately 0.71.

Since the reaction could be easily detected with system A in capped cuvettes, reproducibility studies were carried out for this reaction condition. The decrease in fluorescence intensity upon addition of NAD^+ to the system was found to be reproducible. Figure 16 shows the relative fluorescence intensity vs. time curves for six replicate runs. The curves for all the runs had the same shape, although their starting points were not identical. Two of the curves show more quenching of MB fluorescence intensity. Here, the amount of ADH added to the reaction mixture was less than it was for the other four solutions. This signifies larger MB fluorescence quenching due to a smaller concentration of ADH in the reaction mixture. Further studies need to be conducted to explain this observation and to see the effects of varying ADH, DP and NAD^+ concentrations on the relative size of the signal. As of now, the MB fluorescence decay curves are observed to be reproducible with conventional instrumentation.

MB fluorescence in ethanol vs. in water

Reactions in cuvettes and in the flow system showed a considerable change in MB fluorescence response when it was in ethanol and in water. A short study was conducted using a conventional spectrophotometer to see if the absorbance of MB differs in ethanol and water. Figure 17 shows the absorption spectra of 1.3×10^{-5} M MB in the two solvents. In water, the absorbance maximum of MB is at 664 nm with the relative absorbance intensity of approximately 1.400 a.u. A solution of the same MB concentration prepared in ethanol has a maximum at 656 nm, and the relative absorbance intensity is about 1.794 a.u. Therefore, the absorbance maximum of MB in ethanol is shifted somewhat to 656 nm and yet at 664 nm, which is past its maximum, MB in ethanol still absorbs more strongly than MB in water. This shows the difference in molar absorptivity of MB in ethanol and water.

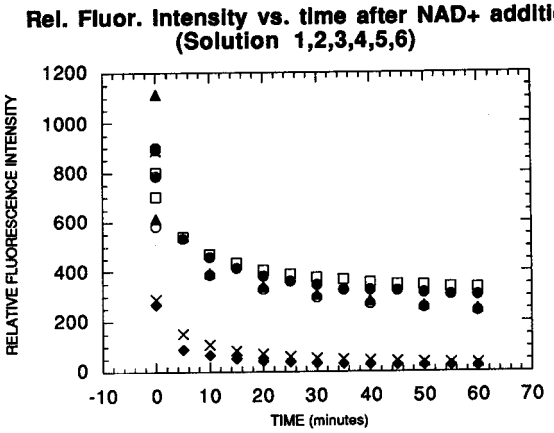


Figure 16: Reproducibility studies for NAD+ addition (system A) in closed, capped cuvettes.

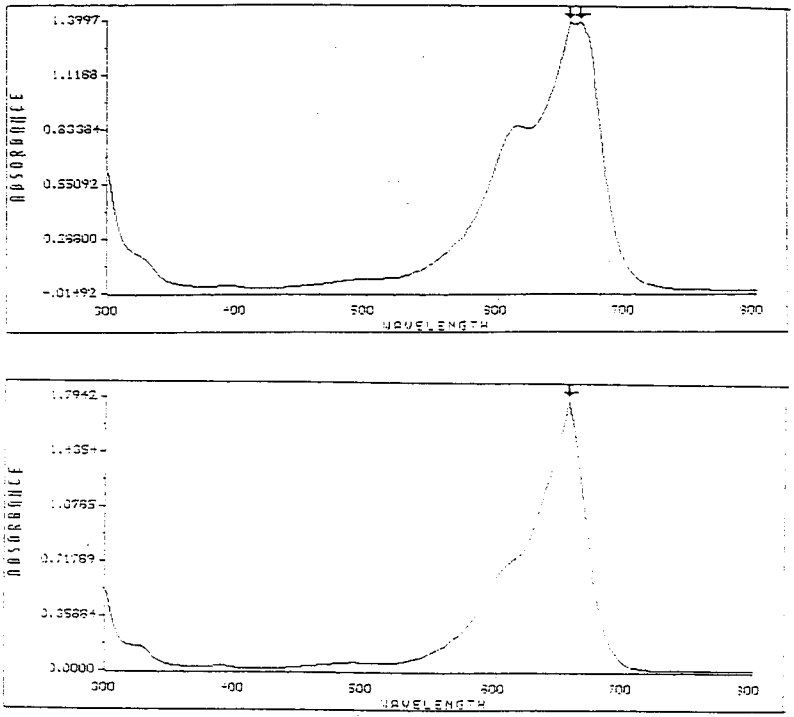


Figure 17: Absorbance spectra of MB in water and in ethanol. Absorbance maximum for MB in water appear at 656-664 nm (1.40 a.u.). Absorbance maximum for MB in ethanol appears at 656 nm (1.79 a.u.).

Reactions conducted on line

After confirming that the reaction can be detected via conventional instrumentation, the reaction was tried on line. Again, ten times the reported concentrations of all the components were used. The experimental set-up was a single-line manifold and the CS was a mixture of all components except NAD^+ (for system A) or absolute ethanol (for system B).

Figure 18 shows the signals observed when NAD^+, water and ethanol were injected into system A. When NAD^+ was injected into system A, a decrease in fluorescence intensity was seen, as expected from the reaction between NADH and MB. When water was injected into this system, again a dip was observed due to the dilution of MB by the addition of water on-line. However, the shapes of the signal due to NAD^+ addition, and water addition were very different. When NAD^+ is added, a wide, blunt dip is seen, indicating an interaction of the components of the reaction mixture. Water addition however, results in a sharp dip, indicating simply a dilution taking place in the flow tubing. When ethanol was injected in the flow system to see what effect it would have on the reaction system A, a peak was observed. This was expected since MB in ethanol has a higher molar absorptivity than MB in water, resulting in a higher fluorescence intensity of MB in ethanol than in water. No reaction was taking place, but ethanol injected into a MB carrier stream gives peaks due to an increase in fluorescence intensity of MB in ethanol, in comparison to water injections.

This result was also seen in system B when ethanol was injected into the system (see Figure 19 a). Upon addition of ethanol, a sharp peak is observed, followed by a small dip. This indicates that some interaction or reaction is taking place. Theoretically, the decrease in fluorescence should be indicated by a dip as MB is converted to leuco-MB. It was observed that ethanol injections gave peaks or dips as signals depending on the concentration of MB used in the reaction mixture. With 5.0×10^{-4} M MB in the carrier stream, injections of 50% ethanol solution gave peaks, but with 5.0×10^{-5} M MB in the

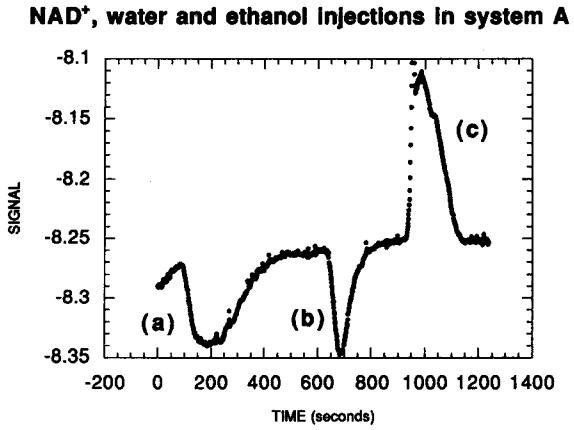


Figure 18: Signal seen in a single-manifold FI system when a) NAD+, b) water and c) ethanol were injected in system A. (Gain = 10^7).

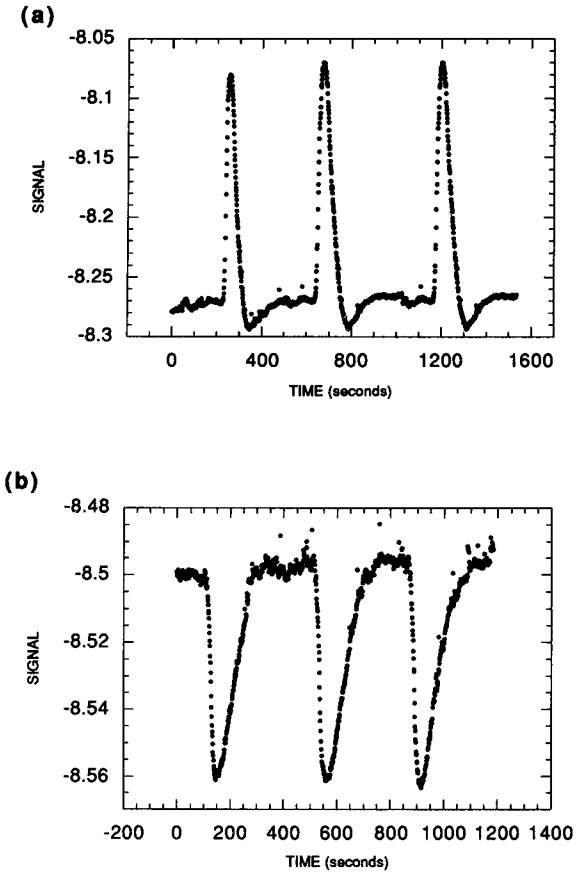


Figure 19: Reproducible signals due to 50% ethanol solution injections in the system with a) 5.0 x 10^{-4} M and b) 5.0 x 10^{-5} M MB in the carrier stream.

carrier stream, 50% ethanol injections in the system gave reproducible dips as signals (see Figure 19). This is because MB, like other fluorescent dyes, does not follow a linear fluorescence emission pattern with increasing concentration of the dye. For example, the concentration dependence of fluorescence intensity of bioacetyl in CCl_4 is shown in Figure 20. At low concentrations, the fluorescence intensity increases linearly with increasing concentration, because absorption is small and emission is proportional to emitter concentration. However, as the concentration increases, the fluorescence intensity levels off and then actually decreases. This decline in fluorescence intensity is observed because absorption increases more rapidly than emission. This process can also be described as a process where emission is quenched by self-absorption. Like bioacetyl, MB also follows this pattern of changing fluorescence intensity with increasing concentration. Thus, if this reaction system for the detection of ethanol is carried out with a high concentration of MB, where MB fluorescence intensity is decreasing with increasing concentration (analogous to point d on Figure 20), then when the concentration of MB is lowered, an increase in MB fluorescence intensity will be observed, as is seen in Figure 19 a. However, if a lower MB concentration is used in the system, then a decrease in MB fluorescence intensity will be observed as a dip, indicating a decrease in MB fluorescence, as seen in Figure 19 b.

For quantitative measurements of injected ethanol in the flow system, varying concentrations of ethanol were injected in the flow system to see if a linear relationship exists between the height of dips, observed as signals for the ethanol injections, and the injected concentration of ethanol. With 5.0×10^{-5} M MB in the carrier stream, 50% ethanol injections in the system gave reproducible dips as signals (see Figure 19 b). However, even with 5.0×10^{-5} M MB in the carrier stream, a linear relationship was not observed with varying concentrations of injected ethanol. For example, 50% ethanol injections did not give signals half the size of 100% ethanol injections. This shows that the concentration of MB used is in a region that is analogous to that between points b and c in Figure 20. Thus, a MB concentration of 5.0×10^{-5} M is in the range where increasing concentration of

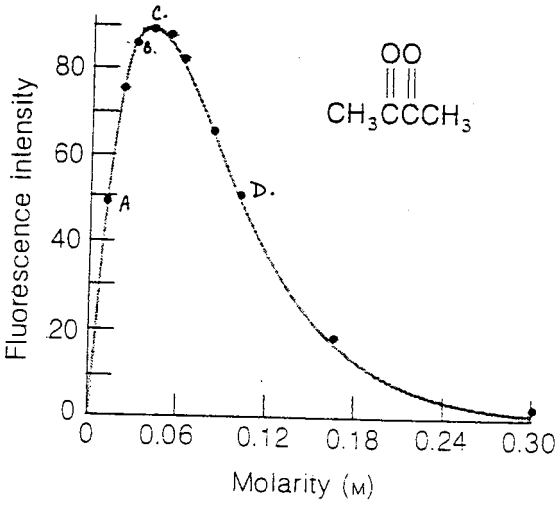


Figure 20: Concentration dependence of fluorescence intensity of bioacetyl in CCl_4 with λ_{ex} = 422 nm and λ_{em} = 464 nm. Reprinted from (4).

ethanol would increase the size of dips seen as signals indicating a decrease in MB fluorescence intensity due to the reaction. However, this concentration of MB is not low enough to give a linear relationship between the size of the signals and the injected ethanol concentration. Further studies need to be conducted with a lower concentration of MB that would allow the signal size to be linearly proportional to the injected ethanol concentration.

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Chapter 4

FUTURE PLANS

Chapter 4

FUTURE PLANS

The goal of this research was to characterize and demonstrate the utility of a compact fluorescence detector for flow injection analysis. This instrument was designed in our lab. To see how our instrument fares in comparison to conventional absorption and fluorescence spectrophotometers, our results for three different reactions could then be compared with results obtained by other researchers for the same chemical systems with conventional instrumentation.

We were successful in carrying out the DMPD reaction system in our experimental setup, and the results obtained can be compared with literature values (1). We can conduct this reaction under less corrosive conditions than Kuban and coworkers (0.100 M vs. 9 M H_2SO_4 in the carrier stream) (1) although our LOD is higher.

The second system we tried was for glucose detection on-line. This reaction system could not be detected successfully in the flow system due to dispersion problems and the long reaction time required. If dispersion can be increased, then perhaps the reaction can take place and be detected on-line. However, as of now, this reaction has only been done in a beaker as a demonstration to show the conversion of MB to leuco-MB, and not in a flowing stream. Pump and flow tubing with larger inner diameter can increase the dispersion and extent of reaction on-line. Such parameters still need to be tested. Perhaps the conversion of MB to leuco-MB requires a longer reaction time (more than 4 minutes). Also, the concentrations of MB in use (1.0×10^{-3} - 10^{-6} M) are much lower than the suggested 1% MB solution for the demonstration in the literature (19). All these factors need to be considered and researched in order to optimize this reaction system.

The third reaction system is designed for the detection of ethanol. This reaction has been observed on-line. However, we have not yet fully characterized this system and used it to detect and quantitatively measure ethanol on-line. Several experiments will be performed. First, to optimize the signal and to see the effects of varying concentration of each of the carrier stream components, different concentrations of ADH, DP, and NAD^+ need to be tested. Further studies need to be conducted to find a carrier stream

concentration of MB for which a linear relationship between the signal size and injected ethanol concentration exists. Solutions of varying ethanol concentrations will be injected in the reaction system with MB concentrations ranging from 5×10^{-6} to 5×10^{-7} M. Calibration curves will be prepared and used to determine the concentration of ethanol in "unknown" solutions.

Therefore, we have been successful in conducting one of the three reaction systems on-line and have obtained comparable data with our instrument in comparison to literature values. Also, the reaction system for ethanol detection has been observed on-line. Further studies need to be conducted with this system for quantitative measurement of ethanol. As of now, our compact and inexpensive instrument has fared well against conventional instrumentation, and additional studies are planned for the future.