Post-Column Electrochemical Reduction Reactor for Fluorescence Detection of Aldehydes Using High Performance Liquid Chromatography

by

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Presented by **MICHAEL J. PATRICK** Y-10-4

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ABSTRACT

Post-Column Electrochemical Reduction Reactor for Fluorescence Detection of Aldehydes Using HPLC

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Some commonly encountered difficulties in High Performance Liquid Chromatographic (HPLC) analyses are the inadequate detectability of analytes in the chromatographic eluent, and overlapping bands resulting in poor resolution of peaks on the chromatogram. These problems make separation, identification and quantization of aldehydes difficult. One common mode of detection frequently used in HPLC, ultraviolet (UV) absorption, is most problematic in this regard. To remedy this problem, a post-column on-line electrochemical reactor was employed, followed by an attempt at on-line fluorogenic derivatization. It was reasoned that using an on-line electrochemical reactor would ensure a controlled, complete, and reproducible reduction reaction, as well as eliminate the need for reagents. Also, concerns of eluent pH were eliminated, since the reaction occurred after the pH-sensitive column.

In this study, a mixture of aldehydes was derivatized with an acidic solution of 2,4-dinitrophenylhydrazine prior to injection onto the chromatographic column. The mixture was injected onto the reverse-phase column using a solution of 0.10 M sodium perchlorate in 55% acetonitrile -45% water as the mobile phase. Immediately after elution from the column, the analytes were electrochemically reduced to 2,4 -diaminophenylhydrazone derivatives, followed by an attempt on-line chemical derivatization using fluorescamine. Detection via a fluorimeter was attempted but was unsuccessful. Using a two-electrode system, on-line electrochemical reduction was successful. A three electrode system employing a Ag/AgCl reference electrode was attempted but was unsuccessful due to the

high pressure of the eluent forcing the reference electrode solution out of the reference electrode housing. Even though fluorimetry and the three-electrode reduction were unsuccessful, use of the two electrode reduction system proved significant. Significant differences between the absorption spectrum of the 2,4-DNPH-aldehyde derivatives and their reduced counterparts were observed. This led to the ability to absolutely identify 2,4-DNPH-aldehyde components in a chromatogram by simply looking at 212 nm/345 nm absorbance ratios.

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LIST OF SYMBOLS USED

a_m	molar absorptivity
c^o	concentration of a substance in bulk of solution
D	diffusion coefficient
dp	particle diameter
E	measured potential in an electrode
Eeq	equilibrium potential in an electrode
e^-	electron
F	Faraday constant
F_m	volume flow rate
fmol	femtomole (1×10^{-15} mole)
H	region of unstablized diffusion regime in a tube
h	reduced plate height
h	entry region in a tube with undeveloped laminar flow
IR	potential drop due to current and resistance (Henry's law)
I_{lim}	limiting current
K	constant dependent upon injection port design
K_o	permeability constant
k	proportionality constant
L	bed length
ℓ	length of tubing or bed reactor
n	number of moles of a compound
n	ground state sigma orbital
Δp	pressure drop
r	radial distance from the center of a tube

r_o	radius of coiling of a tube
R	tube radius
Re	Reynold's number
R_S	resolution of chromatographic bands
T	mean residence time of an analyte in the reactor
T	absolute temperature (in Kelvin)
t_r	retention time of a chromatographic component substance
t_v	residence time
2,4-DAPH	2,4-diaminophenylhydrazone
2,4-DNPH	2,4-dinitrophenylhydrazone
UV	ultraviolet
UV/VIS	ultraviolet/visible region of spectrum
u	interstitial fluid velocity
v	kinetic viscosity coefficient
V_{inj}	volume of injection
V_{det}	volume of detector flow cell
W	width of a chromatographic peak
X	Cartesian coordinate, abscissa axis
x	distance
Y	Cartesian coordinate, ordinate axis
γ	tortuosity factor
λ_1	bed geometry coefficient
λ_2	bed geometry coefficient
η	viscosity coefficient
η	overpotential
κ	coefficient describing the zone broadening in a coiled tube
μ	dynamic viscosity coefficient

μg	microgram (1×10^{-6} grams)
π	mathematical constant = 3.14
π	(pi) a type of bonding orbital that involves overlap of two orbitals.
π^*	a pi orbital of a molecule in an excited state
ρ	fluid density
σ	standard deviation
σ^2	variance
σ^2_{tot}	total variance of chromatographic system
σ^2_i	variance of component i
$\sigma^2_{injector}$	variance of the injector
σ^2_{column}	variance of the chromatographic column
σ^2_{reduc}	variance of reduction reactor
$\sigma^2_{connector}$	variance of connections
σ^2_{mix}	variance of mixing tee
$\sigma^2_{reactor}$	variance of reactor coil
$\sigma^2_{detector}$	variance of detector cell
σ^2_{tr}	variance of the residence time dispersion function in the reaction tube
υ	linear velocity
υ^o	fluid bulk velocity
υ_{max}	maximum fluid velocity

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CHAPTER I

INTRODUCTION

High Performance Liquid Chromatography

Chromatography is a separation method that is based on components partitioning between a stationary phase and a mobile phase in a chromatographic column. The role of the liquid phase is to carry the sample components through the stationary phase. The only time that the components can move through the column is while they are in the mobile phase. The fraction of time a solute spends in the mobile and stationary phases depends upon its distribution coefficient. In order for a separation to occur, the distribution coefficients of the solutes must be different. Chromatographic separations occur because of the relative abilities of the sample components to be adsorbed or absorbed on the stationary phase (1). As a molecule of the sample component enters the column, it can either interact with the stationary phase, or remain in the mobile phase and be carried through the column. A component of the sample that interacts strongly with the stationary phase spends a greater portion of its time within the column than does a component that interacts weakly. Consequently, the retention time, or retention volume (time or volume of solution it takes for the component to be eluted from the column) increases as the amount of interaction on the stationary phase increases (1). The components of the mixture elute from the column as bands and are represented by peaks on the chromatogram. A chromatogram is a plot of the detector signal versus the retention time or retention volume of the eluate.

Chromatographic bands separate because the components move through the columns at different rates. As the length of the column increases, so does the band

broadening or spreading out of the bands. A separation is possible only if the bands move apart faster than they broaden (1). In order to be able to determine the peak area, and thus the relative concentration of the sample component, it is necessary that the peaks be separated or resolved. If there is too much band overlapping, it would be difficult to distinguish where one band begins, and the other ends. The resolution of two bands, R_s, is given by the equation:

$$R_s = 2(t_{rb}-t_{ra}) / (W_a + W_b)$$

where t_{rb} and t_{ra} are the retention times of components b and a respectively, and W_a and W_b are widths of peaks a and b respectively (1). A large value of R_s means that the bands are well resolved.

Since high performance liquid chromatography (HPLC) involves pumping a mobile phase into the inlet of a chromatographic column at pressures as much as 200 times that of atmospheric pressure, a good solvent delivery system is needed. Typically, the technique is done with pressures between 25 and 100 bar (2). A commonly used solvent pump that accommodates these pressures (360 - 1450 psi) is the reciprocating pump. This type of pumping device uses an oscillating piston in a small cylinder that compresses the liquid and pushes it through the system. In order to minimize the pulsating nature of the pump, dual pistons and cylinders are used in alternating cycles, such that one fills while the other one empties (1). The pressure that develops depends on the length of the column, the particle size of the stationary phase, and the viscosity and flow rate of the mobile phase (2).

The diameter of the tubing used in chromatography also has an effect on the pressure. The pressure drop required to maintain a given flow rate increases with the square of the decrease in the diameter of the packing particle or of the column (1). Thus, each two-fold decrease in either diameter results in a fourfold increase in the applied pressure to maintain the flow rate (1).

Reversed Phase Chromatography

In one type of chromatography, reversed phase chromatography, the mobile phase is more polar than the stationary phase. This is usually accomplished by using a hydrocarbon-like bonded stationary phase and a polar solvent for the mobile phase (2). The mobile phase is usually composed of solvents such as methanol, acetonitrile or tetrahydrofuran, mixed with water in varying amounts to get the appropriate polarity for adequate separation of the sample components (2). In reversed phase chromatography, the most polar compounds are eluted first. In order to increase the retention of the solutes in the column, all that is needed is to decrease the polarity of the mobile phase (2). This can be explained by viewing the reversed phase separation process as a partitioning process in which the solutes are distributed between the non-polar stationary phase and the polar mobile phase. The non-polar solutes will be soluble in the stationary phase and will travel through the system more slowly that the polar solutes, which will favor the mobile phase. The distribution can be changed by changing the polarity of the mobile phase. If the mobile phase is made less polar, then the distribution of the solutes is shifted towards the mobile phase, and the retention times decrease.

There are several advantages to using reversed phase chromatography instead of normal phase chromatography (polar stationary phase, less polar mobile phase): it allows for samples containing components of a wide range of polarities to be separated, it uses relatively inexpensive mobile phases, and equilibration of the mobile phase with the column is fast. It can be applied to the separation of ionic or ionizable compounds by using ion pairing or ion suppression techniques, and it is generally faster, easier and more reproducible than other HPLC modes.

Reversed phase chromatography does have its limitations, however. One is the requirement that the pH range of the column be maintained within a range of pH of 3 to 8. If the pH drops below 3, the bonded groups may be removed. If the pH rises above 8, the silica becomes soluble in the mobile phase. If there are any unreacted silanol groups on

the silica surface, it can lead to tailing, excessive retention time, and non-reproducibility between columns due to solute adsorption (2).

Chromatographic Band Broadening

Chromatographic bands are broadened by three kinetically controlled processes: eddy diffusion, longitudinal diffusion, and resistance to mass transfer. Eddy diffusion is a phenomenon that occurs in packed columns because there are many irregular channels between particles. These channels cause solute molecules to travel many different paths through the particles that are not equal in length. This causes solute molecules to reach the end of the column at different times, even though they are traveling in a mobile phase at a constant velocity, which in turn, causes the solute band to spread out. Eddy diffusion, is independent of the flow rate but is dependent upon the column packing material (1).

In longitudinal diffusion, solute bands are broadened because simple molecular diffusion pushes the leading and tailing ends of the band farther apart. This is explained by a natural tendency for molecules to diffuse from regions of higher concentrations to regions of lower concentrations. The rate of longitudinal diffusion is strongly influenced by the viscosity of the solvent, and the temperature. The amount of longitudinal diffusion is controlled by time, thus band broadening increases when the flow rate is decreased (1). Similarly, tubing of a large diameter leads to longer retention times and thus more longitudinal diffusion.

The resistance to mass transfer contribution to band broadening occurs as a solute is partitioning from one phase to the other. The broadening occurs because the phases are not in static contact (and therefore not at static equilibrium) with each other. It is diminished by increasing the speed that equilibrium is reached in the partitioning process. This can be done by increasing temperature, and lowering the viscosity of the solvents (1).

The flow of fluid through the tubing used in the chromatographic system has its own set of dynamics that is of interest in describing chromatography. The Reynold's number, Re, which can be useful in describing fluid flow, is defined as the ratio between inertial and viscous forces in a flowing fluid and the surface of a tube wall. It is mathematically represented as $Re = \rho v^0 \ell / \mu = v^0 \ell / \nu$, where ρ is the fluid density, v^0 is the velocity of the bulk fluid, ℓ is the length of the tube, and μ is the dynamic viscosity coefficient of the fluid (3).

On passage through a "...tube, boundary layers develop at the walls and their thickness increases until they merge at a certain distance, h, from the inlet", where h is the entry region within a tube of undeveloped laminar flow (3). A steady state is established with a parabolic velocity profile that is given by: $v(r) = v_{max} (1 - r^2/R^2)$ where R is the tube radius, and r is the radial distance from the tube center (3). v_{max} is given by $R^2 \Delta p / 4 \mu x$, where Δp is the pressure drop across the tube, μ is the dynamic viscosity coefficient of the fluid, and x is the distance traveled (3). Figure 1 shows how the velocity profile changes from rectangular at the inlet of a tube to parabolic at some distance, h, from the inlet.

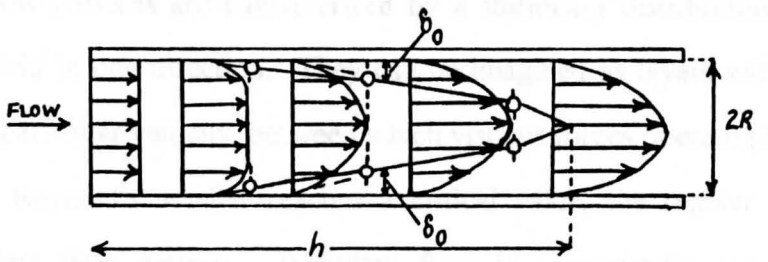


Figure 1: Laminar Flow Through a Tube. R -- tube diameter, h -- entry region (Adapted from reference 3, p. 40).

The distance from zero to h is the entry region or the region of undeveloped flow pattern, and its length is given by: $h \sim 0.04\ R\ Re$, where R is the inner radius of the tube,

and Re is the Reynold's number. In Figure 2, the stages of development of laminar flow are shown, with the origin of the dispersion noted.

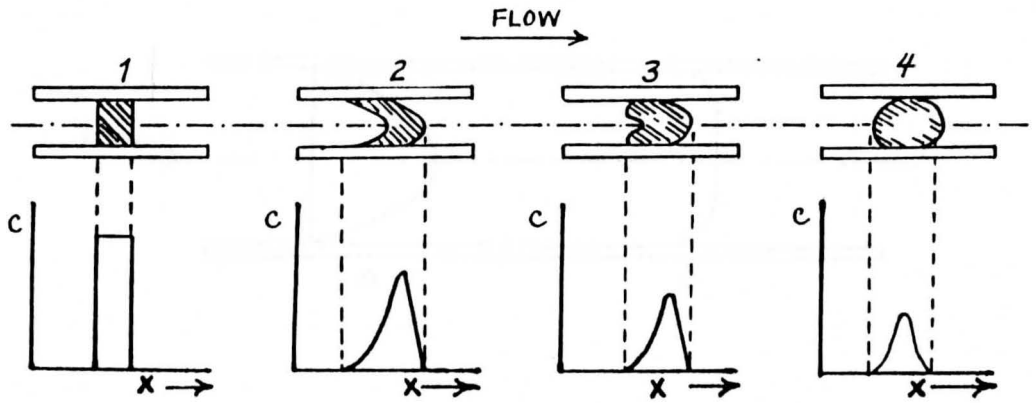


Figure 2: Dispersion of Rectangular Zone of a Substance in a Laminar Flow. " *1* -- Initial stage, no dispersion; *2* -- early stages of dispersion, controlled by convection (parabolic velocity profile); *3* -- dispersion at later stages, controlled jointly by convection and diffusion; *4* -- late stage of dispersion, with diffusion predominating" (3). (Adapted from reference 3, p. 54).

Laminar flow patterns are characterized by a stationary distribution of the local velocities of the fluid in one direction. They can be imagined as layers within the fluid, each with its own particular velocity, between which viscous forces operate (3). When the magnitude of the Reynold's number reaches a critical value, the regular flow pattern becomes a turbulent flow pattern. Turbulent flow is a stochastic process and the trajectory of a certain particle in the fluid becomes random (3).

Turbulent flow within a tube consists of random flow pulsations containing a variety of velocities and amplitudes. The average flow produces a net overall flow in a certain direction. There is intense mixing within turbulent flow that is not found within laminar flow (cf., the laminar layers do not mix). The average velocity distribution of

turbulent flow in a tube is logarithmic with the maximum velocity at the center of the tube (3). A comparison of laminar and turbulent velocity profiles is shown in Figure 3.

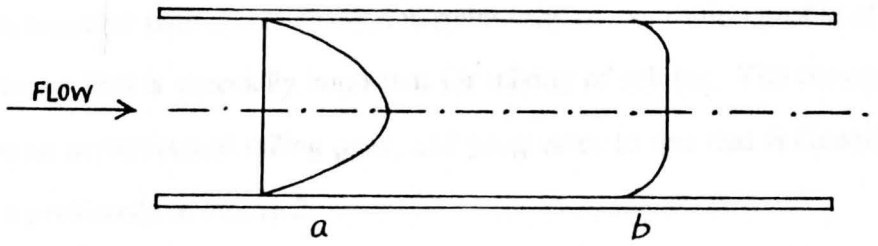


Figure 3: Laminar and Turbulent Flow Velocity Profiles. (a) Laminar flow; parabolic velocity profile. (b) Turbulent flow; logarithmic average velocity profile. (Adapted from reference 3, p. 43).

Turbulent flow is undesirable in practical measurement systems such as HPLC because of poor reproducibility and the impossibility of applying a rigorous theoretical treatment. However, in some cases turbulent flow can be advantageous, such as when intense mixing of the fluid is desired, resulting in increased mass transport and equalization of local velocities (3).

The situation is slightly different when the fluid percolates through a chromatography column or bed reactor rather than through an open tube. The Reynolds number is defined by

$$Re = \upsilon^0 dp/v$$

where υ^0 is the fluid bulk velocity, dp is the particle diameter, and v is called the kinematic viscosity coefficient. The Reynold's number is the ratio between the inertial and the viscous forces in the flowing fluid. Turbulence develops gradually as Re increases from 1 to 100. Irregularities or pronounced curvature of the surface touching the fluid may lead to formation of local turbulence at a substantially lower Reynolds number. Turbulence is

thus induced in tubes by coiling, twisting, or squeezing them. The resulting local velocity equalization can be used to suppress the analyte band broadening in a liquid stream (3).

There are two types of diffusion that occur within a solute band flowing in a tube: axial and radial. The axial (or longitudinal) diffusion can generally be neglected. Radial diffusion, together with convections, determines the concentration profile of a solute in the fluid stream. This is especially important for mixing of solutes. The concentration profile begins as an asymmetrical tailing peak, and progresses to one that is Gaussian shaped (3), as shown previously in Figure 2.

The elution of a chromatographic band produces a Gaussian concentration profile at the detector, which results in a Gaussian shaped peak on the chromatogram. With the assumption of a Gaussian shape, the width of the resulting chromatographic peak can be expressed in terms of the peak standard deviation, σ. When considering a complex system in which each component contributes a fraction to the overall band broadening, statistical independence is assumed (3). Therefore, the overall variance, σ^2_{tot}, of all the individual contributions to band broadening (widening of the Gaussian peak) are simply additive:

$$\sigma^2_{tot} = \Sigma_i \sigma_i^2$$

Individual contributions to band broadening are assumed only when a single factor predominates overall. If several factors contribute, they must be considered together as a single term. However, it is possible to discuss each of the effects separately. The system developed for this study is a good example of a complex system whose band broadening is the sum of its component variances (3):

$$\sigma^2_{tot} = \sigma^2_{injector} + \sigma^2_{column} + \sigma^2_{reduc} + \sigma^2_{connectors} + \sigma^2_{mixer} + \sigma^2_{reactor} + \sigma^2_{detector}$$

where the subscripts refer to where the band broadening occurs (reduc is reduction reactor).

With regard to each of the components being handled separately, the injector introduces a variance of $\sigma^2_{injector} = V^2_{injector}/K^2$, where V is the volume of injection, and K is a constant that depends on the injection port design. If the injected zone is

considered to be rectangular in shape, then $K^2 = 12$. It is also assumed that the sample is not mixed with the mobile phase (3). The broadening in a piece of straight tubing such as a connector is given by: $\sigma^2_{connector} = \pi R^4 \ell F_m / 24D$ where R is the inner radius, ℓ is the length of the tubing, F_m is the volume flow rate, and D is the diffusion coefficient. Thus, band broadening in a connector can be minimized by using small inner diameter tubing (3). Dispersion increases with increasing flow rate and decreasing diffusion coefficient of the analyte solute. The dispersion of the analyte zone can be decreased by creating additional flow in the tube and thus equalizing the local velocities within the liquid. This is achieved by coiling, squeezing or distorting the tube. The value of σ decreases on coiling of the tube, in direct proportion to the value of a dimensionless parameter $(2R)^2\rho\upsilon^2/\mu r_0 D$, where R is the inner radius of the tube, ρ is the density, υ is the linear velocity of the mobile phase, μ is the mobile phase dynamic viscosity coefficient, r_0 is the radius of coiling of the tube and D is the diffusion coefficient of the solute within the mobile phase (3).

The detector contributes to band broadening by: $\sigma^2_{det} = V^2_{det} / F^2_m$, where V is the volume of the flow cell, and F_m is the volumetric flow rate. The variance produced by the detector depends mainly on the volume and the geometry of the detection cell, and by the time constants for response of the detector (3). In this discussion, the rectangular shape of the solute zone is maintained, and the effective volume equals the cell volume.

Pre-Column Derivatization

Chemical modification of an analyte by pre-column derivatization can facilitate and improve the analysis of that analyte. Chemical derivatization can be carried out prior to its introduction to the chromatographic column, or immediately following the elution of the sample band from the column. According to Sternson (4), pre-column derivatization, when introduced into an analytical scheme, allows for: enhancement of the stability of the analyte; improvement of the separation of the analyte from the sample matrix; refinement

of the subsequent chromatographic separation by improving band shape and/or increasing the resolution; and the improvement of detectability by increasing response to the detector or by introducing an additional element of specificity into the determination due to the limited reaction possibilities of the reagent (4). Obviously, no effect on band broadening occurs prior to the column.

In the selection of a chemical reaction that is to be used in a pre-separation derivatization scheme, the chosen reaction must be rapid, quantitative and/or reproducible; convert the analyte to a single product, utilize reagents that can be separated from the final product; and form derivatives that have detection properties different from the reactants while preserving the ability to chromatographically separate components. Reactions need to be optimized with regard to solvent, temperature, reaction time, and susceptibility to catalysis. The solvent should be selected so that it allows the most favorable reaction conditions, but also so that it is compatible with the chromatography (4).

Pre-separation derivatization reactions usually involve oxidation, reduction, displacement, or addition reactions. Of the most concern here is the addition reaction. In general, aldehydes undergo nucleophilic addition reactions, with the nucleophile attacking the carbon atom of the carbonyl group, and the electrophile reacting with the oxygen atom of the carbonyl group (4). Derivatization of aldehydes is primarily done in conjunction with HPLC to improve detectability, but it also stabilizes the aldehydes, by interfering with their relatively easy oxidation to carboxylic acids. The two-step reaction of the aldehyde with a nucleophile to form the derivative product is controlled by pH, which controls the reaction by kinetics (4). Such a reaction is shown in Figure 4.

Using the reagent 2,4-dinitrophenylhydrazine to improve the detectability of aldehydes and ketones is a common practice. The reaction with aldehydes forms a chromophoric hydrazone, which can be detected by UV spectrophotometry (4).

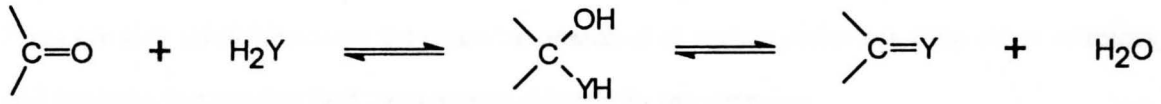


Figure 4: Reaction of an Aldehyde with a Nucleophile, Y.

The greatest advantage of using precolumn derivatization over post column derivatization is the greater freedom and flexibility in the selection of the reaction conditions, which means there are fewer conditions imposed upon the reaction kinetics. Also, there is usually less need to--and less difficulty in--removing excess reagents prior to detection (4).

The disadvantages of pre-column derivatization are the possibility of formation of multiple products from one analyte, and formation of artifacts that may interfere with the determination. Pre-column derivatization is most commonly used to extend the utility of sensitive HPLC detectors to compounds that are relatively insensitive to them, by chemically transforming those compounds into species that are more readily detectable (4).

Post-Column Reduction (Bed Reactor)

If one desires to derivatize after a chromatographic separation, bed reactors are best for reactions of intermediate speed and residence times of 30 sec up to several minutes. A packed bed reactor is essentially a regular chromatographic column that operates under non-retention conditions. The geometry of a packed bed is the most important consideration, using the same type of column packing techniques that are employed in packing an HPLC column. The bed reactor is not as commonly used as other derivatizing methods in chromatography, but it does have its advantages. It is particularly well suited for certain types of reactions such as those having intermediate kinetics (4).

They are also useful because they can be operated at higher pressures than other reactors, and because the reactor bed participates directly in the reaction.

Effects on band broadening and pressure drop can be expected as a function of reaction time. Band broadening can be explained by such factors as axial molecular diffusion and convective mixing. It can be predicted by using an empirical equation proposed by Hilby et al. (4):

$$H = (L\Delta\sigma^2_{tr})/t_v^2 = 2\gamma D/u + \lambda_1 d_p/[1+\lambda_2(D/ud_p)^{1/2}]$$

where L is the length of the bed, $\Delta\sigma^2_{tr}$ is the variance of the residence time dispersion function in the reaction tube, t_v is the mean residence time, γ is the tortuosity factor, D is the diffusion coefficient, u is the interstitial fluid velocity, λ_1 and λ_2 are bed geometry constants, and d_p is the mean particle diameter (4). The pressure drop for a bed reactor would be lower than that for a tubular reactor, and is given by:

$$\Delta p = u\eta L / K_0 d_p^2$$

where u is the interstitial fluid velocity, η is the viscosity coefficient, L is the bed length, K_0 is the permeability constant and d_p is the particle size (4).

The variance is $\sigma^2_{bed} = T^2 hdp/\ell$, where T is the mean residence time of the analyte in the reactor, h is the reduced plate height, dp is the particle diameter, and ℓ is the length of the reactor (3).

Mixing Units (Tee's)

Introduction of another solution into the chromatographic eluent requires a device that will allow adequate mixing without producing large amounts of band broadening. Mixing units in flowing systems are commonly known as mixing tees. Mixing ratios and solution composition, such as viscosity and density, play an important role for optimal construction and geometry of mixing tees (4). The mixing of the reagent and the eluent

streams is of primary concern prior to reaching the detector and mixing should be complete before the fluid stream enters a reactor. A narrow coiled tube gives the best radial mixing and, as noted earlier, is better than longitudinal diffusion for bringing the added solution in contact with the substance flowing in a tube (3,4).

Certain geometries provide better radial mixing than others. Generally geometries are measured in comparison with the angle the addition path makes with the eluent path. Since the flow is turbulent, as characterized by sudden changes in flow path as introduced by the geometry of the mixing tee, larger amounts of band broadening may be introduced depending on the geometry chosen. For example, the band broadening produced from using a mixing tee of 90 degree orientation is greater than that of 30 degree orientation (4). The feasibility of using a particular mixing tee can be judged by the mixing it induces versus the band broadening it causes.

Post-Column Reaction Detector

There are several advantages to using a reaction detector in liquid chromatography, which were noted earlier in the discussion of pre-column derivatization. The main reason is for specificity of reaction, and improvement of detection limits. However, there are some major disadvantages that go along with the bonuses gained by using a reaction detector in chromatography. One of these is the influence of the mobile phase on the reaction medium. It is a rare occurrence that the optimal chromatographic eluent is the best medium for the reaction of interest (4). The major advantages in using a post-column reaction detector are the absence of artifact formation, and the fact that it is not necessary for the reaction to go to completion or be well defined. The only requirement is that there be good reproducibility of the reaction (4).

Another concern of reaction detectors is the kinetics of the reaction (4). It is obvious that a detector reaction must occur rapidly. Associated with this is the reactor

design, which must attempt to reduce band broadening and affect resolution as little as possible. In an attempt to accomplish maximization of mixing, and minimization of band broadening, a commonly used reactor is a straight or helically coiled tube made of different materials such as glass, Teflon, or steel, and of widely differing geometries (4). In this type of reactor, the solvent streams are non-segmented, and the flow patterns are predominantly laminar. The band shapes encountered in this reactor are as would usually be expected in a linear reaction tube (see Figure 2). That is, they usually start from a plug injection with a square shape and "...a peak profile is then obtained whose deviation from a Gaussian profile is an indication of the geometrical quality of the reaction design" (4). There are two phenomena responsible for these concentration profiles: axial dispersion and axial diffusion. The axial dispersion results from velocity gradients in the laminar flow patterns. Frei explains this phenomenon (4): "...the velocity of the fluid stream in the center is twice the mean velocity of the stream and at the layer closed to the tube wall it would essentially be zero." The axial diffusion is usually negligible when compared to the dispersion caused by the velocity gradient. However, "...molecular diffusion from the wall to the center will tend to effectively counteract the formation of the velocity profile and in narrow tubes will result in a decrease of the dispersion when going to lower flow rates" (4). Band broadening in the reaction detector is composed of the individual contributions from connections, mixing units, the reactor unit (coiled tube), and the detector cell.

The analyte zone broadening is expressed by $\sigma^2_{reactor} = \kappa T(2R)^2/96D$, where κ is a coefficient describing the zone broadening in a coiled tube (and is greater than 1), T is the mean residence time of the analyte in the reactor, R is the inner radius of the tube, and D is the diffusion coefficient. The smaller the tube radius is, the less the broadening. However, the pressure drop increases according to Poiseuille's equation: $\Delta p = 512\mu F^2_m T/\pi^2(2R)^6$, where μ is the dynamic viscosity coefficient, F_m is the volume flow-rate, T is the absolute temperature, and R is the inner radius (3). Open tube reactors are generally suited for fast reactions (retention time of less than 30 seconds).

Electrochemical Reduction Reactions of Organic Compounds

In order for all chemical and electrochemical reactions to occur, movement of electrons must occur. Oxidations occur at the positive electrode (anode), and reductions occur at the negative electrode (cathode) in an electrolysis cell. Electrochemical reductions involve a gain of electrons by passing an electric current through a solution that contains the reducible chemical species via two electrodes. The working electrode is the electrode at which the reaction of interest occurs -- for this study a reduction (gain of electrons). This electrode is accompanied by an auxiliary electrode, which functions as the opposite electrode -- for this study an oxidation (loss of electrons).

The fundamental event in electrodic reactions is the electron exchange at the electrode-solution interface. This interface is called the electrical double layer, and is a very narrow region under the influence of enormous electrical fields. Three things must occur for an electroorganic reaction to occur (5):

1. The transfer of the organic species to the electrode surface.
2. Exchange of electrons between electrode and organic species (may involve adsorption).
3. Removal of the primary products from the electrode surface. (may involve desorption).

During an electrochemical reaction, the potentials of the electrodes in the system are not in a state of equilibrium due to the passing of an electric current, which tends to displace the potentials of the electrodes. Under the conditions of an electrolytic oxidation or reduction, the electrodes are polarized. A depolarizer can be added to reestablish the equilibrium state (i.e., no polarity). So, for a reaction to occur, the polarizer (electrodes with current) and the depolarizer (the substances in solution that can be oxidized or reduced) must be present (6).

According to Kyriacou, in *Basics of Electroorganic Synthesis,* the electrode potential is the "...difference, in volts, as measured against a reference electrode, at an

electrode-solution surface" (5). There are two methods that are used to attain the desired electrode potential. The first is to use an electrode constructed of a material of that has the proper overpotential. Overpotential, or overvoltage, is the difference between the measured and equilibrium electrode potentials (5). Mathematically, this is represented as $\eta = E - E_{eq}$, where η is the overpotential, E is the measured potential (versus a reference), and E_{eq} is the equilibrium potential of an electrode (7). For the hydrogen evolution reaction the hydrogen overpotential refers to the cathodic reaction:

$$2H^+ + 2e \longrightarrow H_2$$

for the oxygen evolution reaction the oxygen overpotential refers to the anodic reaction:

$$2H_2O \longrightarrow 4H^+ + O_2 + 4e$$

The higher the hydrogen overpotential, the more powerful is its reducing ability (6).

A second method used to attain the desired electrode potential involves the use of a high hydrogen overpotential, and an electrode voltage controlled by an applied voltage, as measured by a reference electrode of known potential (6).

Electrolytic Cells

There are two types of electrolytic cells, those that employ two electrodes: working and auxiliary electrodes; and those that employ three electrodes: working, auxiliary and reference electrodes. The working electrode is the electrode at which the reaction(s) of interest takes place. The auxiliary electrode maintains the balance of electrons in the solution by performing the other half of a redox reaction on another reactible substance(s) in the solution. The main disadvantage of the two electrode system is that it is difficult to know precisely the actual potential present at the surface of the working electrode. This failing is remedied, however, by including a reference electrode as the third member of the measurement system. The reference electrode is constructed so that it is of known constant, potential. The potential difference between the reference and

auxiliary electrodes is compared to the difference in potential between the working and auxiliary electrodes. From this comparison, the working electrode potential can be well controlled using a device known as a potentiostat. The reference electrode must be placed in close proximity to the auxiliary electrode to minimize *IR* drops (*I* is the current, *R* is the resistance to electron flow) (7).

A commonly used reference electrode is the Ag/AgCl electrode, in which the Ag^+ ion is converted to Ag metal in a KCl saturated solution (7). The chemical reaction is shown below:

$$AgCl\,(s) + e^- \rightleftharpoons Ag\,(s) + Cl^-\,(aq)$$

The electric potential for the reaction is $E^o = +\,0.222$ V.

Electrolytic processes that occur at non-zero net current can be classified as controlled potential techniques (7). One of interest is coulometric operation of the electrochemical cell in which the potential is maintained constant with respect to the reference electrode. This allows for control of the completion of the electrochemical reaction (7). In such a process, all of the electroactive substance is converted (reduced or oxidized) at the working electrode, and the electric charge being passed is proportional to the total amount (3).

There are several other variables besides the electric potential that can be changed to affect the electrochemical reaction. They generally are the same as those that affect regular chemical reactions and include: temperature, protic or aprotic solvents, pH, concentrations of reactants, relative amounts, methods of mixing, and reaction time (5). In addition to these common variables, electrochemical reactions introduce other types of variables: the electrode potential, the electrical double layer, the electrode material, the concentration and kind of the supporting electrolyte (which affects the conductivity and accessible potential range of the medium), the structure of the electrical double layer,

nature of the final products, the type of cell, (divided or undivided), the physical composition of the electrolysis medium, and the degree and means of agitation (5).

With regard to electrode material selection, almost any electronic conductor can be used in the form of plates, rods, wires, gauzes, wool, sheets, mercury pools, and others (5). Alloys may be used also. The electrode material is of significance, but the main concern is the microscopic surface morphology, because it is only the surface of the electrode, which is in contact with the medium, that is the actual electrode. Electrode materials that are best for direct electrochemical reductions are the soft metals: lead, cadmium, zinc, tin and mercury, because of their high hydrogen overpotential. The solvent medium must be able to conduct an electric current. Therefore, it must contain at least one component of high dielectric constant that can dissolve and also ionize sufficient amounts of a salt, an acid, or a base, for the medium as a whole to be conductive (5).

These ionizable substances are called supporting electrolytes and their function is to carry the current through the solution. Solutions that are poor conductors of electric current must contain such an electrolyte. A depolarizer is a substance that is preferentially oxidized or reduced at the auxiliary or counter electrode (5).

The specific reduction of interest here involves the chemical reduction of nitro groups ($-NO_2$) to amino groups ($-NH_2$). The nitro group is one of the best electrophores in regard to both ease of reduction and versatility of derived products. In acid solutions and at mercury or lead cathodes (high overpotential electrodes), amines are mostly produced (5). There is an advantage to doing this reaction electrolytically: it enables the selection of the potential of the cathode (the reducing power) so as to direct the course of the reaction toward the desired objective. This reaction is a three step process (6), as shown in the following equation:

$$-NO_2 \xrightarrow{2e^-} -NO \xrightarrow{2e^-} -NHOH \xrightarrow{2e^-} -NH_2$$

Each of the above steps is controlled by the potential at the cathode surface (6). Divided cells are required because the reduction products are susceptible to anodic

oxidation (5). Various reductions are possible at all pH's, but the final products depend upon pH and electrode potential (5). Some of the possibilities are shown in Figure 5.

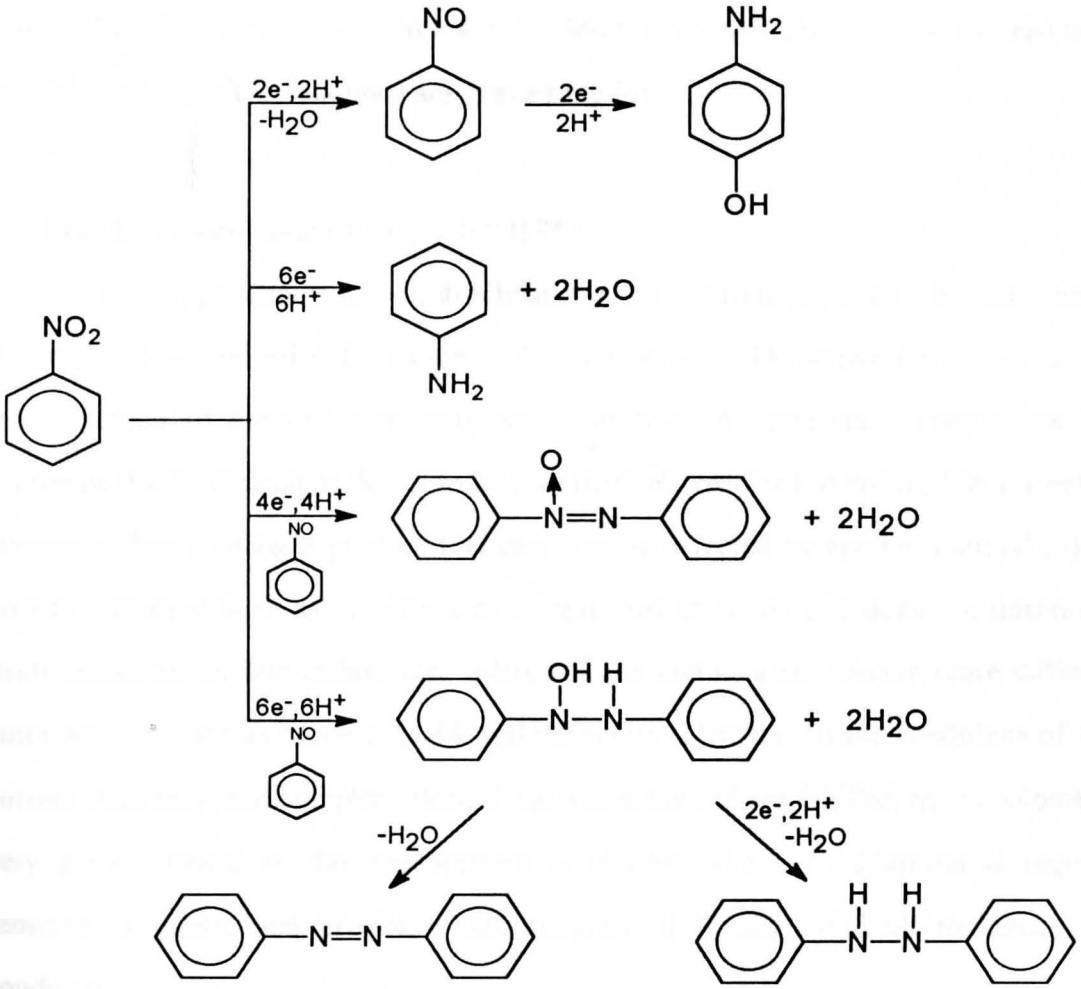


Figure 5: Possible Products of Nitro-group Reduction on Benzene (5).

In the special case of reducing 2,4-dinitrophenylhydrazones to 2,4-diaminophenylhydrazones, there are two nitro groups, which are reduced separately (6). To break these steps down, we have:

$$-C_6H_3NO_2 + 2H^+ + 2e \longrightarrow -C_6H_5NO + H_2O$$

$$-C_6H_5NO + 2H^+ + 2e \longrightarrow -C_6H_5NHOH$$

$$-C_6H_5NHOH + 2H^+ + 2e \longrightarrow -C_6H_5NH_2 + H_2O$$

However, the presence of more than one nitro group may lead to complex mixtures (6). The amount of potential applied determines the extent of reaction or how many of the above steps are completed. Most processes that involve the electrolytic reduction of organic compounds are irreversible (6).

On-Line Electrochemical Reactor for HPLC

One application of electrochemistry to chromatography is the use of electrochemical derivative formation in flowing liquids. Derivative-formation reactions are employed to improve sensitivity and selectivity of detection. They allow for a substance that is difficult to detect to be quantitatively oxidized or reduced at a generating electrode, thus forming a product that can then be detected by another method (3). An on-line technique such as this is not as complicated or costly and does not introduce as many errors as off-line techniques. Also, off-line methods are usually more difficult to automate (3). Of this process, Stulik and Pacakova (3) write, "If the conditions of 100% current efficiency, and complete electrolysis within the cell are fulfilled, then coulometry is very advantageous, as the measurement is absolute (i.e. no calibration is required), sensitive, accurate, and precise. Unfortunately, it is often difficult to satisfy these conditions."

Although electroanalytical methods are characterized by high sensitivity, good precision and accuracy, there are some limitations. One of these is the carrier liquid, which must have sufficient electrical conductivity, and also yield an electric signal that is constant and as small as possible. In other words, it limits the solvents for reaction to those that have sufficient polarity, or to relatively non-polar solvents that are dissolved in water. Another limitation is the difficulty in maintaining a constant and reproducible electrode surface activity (3). This is because when an electrode is in contact with a solution, various processes can occur at the electrode surface, such as solute adsorption

and/or catalytic effects, redox reactions other than the one of interest, or ion-exchange, which can cause continuous changes in the electrochemical properties of the electrode. The above phenomena usually result in electrode passivation (3). However, when used in HPLC, the problems related to electrode passivation are usually substantially less because in the flowing system, the working electrode is continuously being washed with pure carrier liquid, and is exposed only occasionally to short zones of passivating substances throughout the experiment (3).

In the design of the tubular electrochemical reactor of length ℓ, and radius R, the limiting current equation is $I_{lim} = 2.01nFD^{2/3}\ell^{2/3}\upsilon^{2/3}kR^{2/3}c^{o}$, where n is the number of moles of the compound, F is the Faraday constant, D is the diffusion coefficient, υ is the linear velocity, c^{o} is the concentration of a substance in the bulk solution, and k is a proportionality constant, where k = 0.33 for laminar flow, and k = 1.0 for turbulent flow (3).

Use of an on-line electrochemical reactor allows for selectivity in the reduction process since, unlike chemical reduction, the reduction potential of the electrode is adjustable (8). "Microcoulometric designs can be used to directly oxidize or reduce analytes on the electrode surface, to produce more easily detectable species for fluorescence or electrochemical detection (9)."

Some disadvantages in using a reductive system for detection include reduction of traces of oxygen, hydrogen ions and trace metals in the solution. This requires removal of oxygen from the mobile phase and the samples, or coupling it with a method that introduces an element of specificity to reduced compounds. The potential at which certain compounds are reduced does some of this (3).

If water is in the reaction medium, it will give a more efficient reaction because the resistance of the solution will be diminished, and will require less power to complete the reaction, and therefore will evolve less heat (6).

Ultraviolet detection

Ultraviolet (UV) Absorbance detectors used in HPLC generally use either a mercury vapor or a deuterium discharge lamp as a light source. The greatest spectroscopic sensitivity is obtained using a Hg lamp and monitoring absorbance at 254 nm. Use of a deuterium lamp gives less sensitivity but allows wavelengths other than 254 nm to be monitored. Additional specificity of analysis can be introduced by forming derivatives that absorb above 300 nm. This is advantageous because there is a more limited range of materials that absorb light at these longer wavelengths (4).

Spectrophotometric detection of chromophoric species in the ultraviolet range is a useful and common mode of detection when separating ultraviolet light absorbing compounds by HPLC. UV detection is a readily available and simple mode of detection. Spectrophotometric detectors are rugged and generally unaffected by external influences (4). Of the specificity of UV detectors, Frei and Lawrence write in *Chemical Derivation In Analytical Chemistry*, "...their specificity depends on the relative ability of other compounds in the analysis mixture to absorb UV light at the wavelength at which the column effluent is being monitored" (4). Sensitivity is another desirable quality of a detector. In UV detectors, sensitivity is "...dictated by the molar absorptivity, a_m, of the compound of interest" (4). Most UV detectors can detect to levels of ~ 1 μg of a substance that has an a_m value equal to ten (4).

Molecular absorption in the ultraviolet and visible regions of the spectrum depends upon one thing: the electronic structure of the molecule involved. Usually, those molecules containing conjugated systems [i.e., those in which the location of π orbitals allow it to overlap other orbitals in the molecules (10)] readily absorb ultraviolet energy (11). When energy is absorbed by a molecule it is quantized or grouped in discrete bundles. This increase in energy results in the elevation of electrons from lower energy orbitals in the ground state to higher energy orbitals in an excited state (11). UV/VIS absorption spectrophotometry is thus a generally useful tool for HPLC detection because

many compounds contain functional groups that will absorb light well in these regions of the spectrum.

A diode array spectrophotometer monitors all wavelengths in the UV/VIS spectrum at once. When coupled with chromatography, it allows for a UV spectrum to be taken at specific time intervals. This allows the chromatographer to determine the wavelength of maximum absorbance at any retention time along the chromatogram. Thus, the wavelength of maximum absorbance for a component of particular interest can be selected. This would maximize the detector response for that component, resulting in larger detection peaks, and may minimize unwanted component detection that may have a less than maximum wavelength at the one selected.

Fluorescence detection

Fluorescence detectors offer an extra element of specificity and sensitivity beyond that of UV detectors. This is primarily due to the fact that they directly measure emitted light rather than the difference between the incident and transmitted light. The extra sensitivity is introduced by the ability to select excitation and emission wavelengths that are far removed from one another. Fluorescent molecules are characterized by double-bond conjugation, and substituents directly on an aromatic ring that promote $\pi \longrightarrow \pi^*$ electron transitions within the molecule (4). Fluorescence intensity is decreased by introducing electron attracting groups, such as nitro or carbonyl groups, in conjunction with the aromatic system. This promotes $n \longrightarrow \pi^*$ electronic transitions, which results in increased intersystem crossing (4).

Fluorescence is a very sensitive method of detection. The theory involves the basic principles of luminescence. Luminescence occurs when an electron that is in an excited energy electron level falls into a lower energy level with the simultaneous emission of radiation (12). An electron configuration at the lowest energy levels possible is in the

ground state and is very stable. In order for luminescence to occur, one or more electrons that are in the ground state (lowest energy level) must be excited to a higher one. This can be done by absorbing light energy in the ultraviolet region. During the absorption process, the spin of the electron does not reverse. If a molecule is in the excited state, it normally loses energy rapidly through radiationless processes such as vibrational relaxation and internal conversion until it arrives at the lowest vibrational level of the first excited singlet state (12). If the excited electron returns to the ground state while simultaneously emitting energy that is equivalent in energy to the energetic difference between the excited and ground states, fluorescence occurs. The spin of the electron does not change during the transition.

It is sometimes possible for either the unpaired ground-state electron or the excited state electron to reverse its spin, while still in the excited state. In this case, the excited electron has a spin that is identical to that of the unpaired electron in the ground state. A molecule that has an unpaired electron in an excited state that has a spin that is identical to that of another unpaired electron in the molecule is in the triplet state.

The radiation that is emitted is at a wavelength that is characteristic of the energy level differences between the electrons, and corresponds to radiation in the ultraviolet or x-ray region. In solutions, the fluorescent radiation is of lower energy than the absorbed radiation, that is, the fluorescence radiation occurs at a wavelength that is longer that the wavelength of the absorbed radiation. Fluorescence occurs nearly instantaneously after absorption of energy, usually from 10^{-6} to 10^{-10} seconds, which is slow compared to the 10^{-15} sec for absorption to occur (12).

CHAPTER II

HISTORICAL

Aldehydes: Uses and Properties

Aldehydes are a class of compounds that are very reactive and are both biologically and industrially important. In industrial processes, aldehydes are reduced to make the corresponding alcohols, and are used to manufacture dyes, resins and various plastics. Some aldehydes are ingredients in perfumes and flavoring agents. One aldehyde in particular, formaldehyde, is widely used industrially in the manufacture of resins, chelating agents, and industrial chemicals (13). In residences, aldehydes are found in plywood and particle board, insulation, combustion appliances, and tobacco smoke. Aldehydes are released in motor vehicle exhaust as hydrocarbon fuels burn incompletely. Methanol powered automobiles are a source of formaldehyde, due to significant formation of formaldehyde in the combustion of methanol (14).

An aqueous solution of formaldehyde, called formalin, is used in antiseptics, fumigants, tissue and biological specimen preservation, and embalming fluid (13). Because of it's effectiveness as a sterilizer, it is commonly used to sterilize kidney dialyzers and tubing (15). Formaldehyde and acetaldehyde are also present in mainstream cigarette smoke (16). Although aldehydes are produced by natural processes within the human body, they are toxins. In animal experiments, formaldehyde has been shown to be a lung carcinogen. In general, the lower molecular weight aldehydes attack exposed moist tissue, particularly in the eyes and mucous membranes of the upper respiratory tract. This attack on moist tissue is facilitated by the high water solubility of aldehydes, and their intensely irritating qualities. Acetaldehyde, in particular, acts as an irritant and systemically as a narcotic to the central nervous system (13). Metabolically, aldehydes are converted to the

corresponding carboxylic acids. Formaldehyde and other aldehydes are receiving attention as promoters in the photochemical formation of ozone in the atmosphere (17).

Chromatographic Separation Of Aldehydes

On the subject of separating, identifying and quantifying aldehydes, there are numerous chromatographic methods. Historically, thin layer chromatographic methods were used, and were eventually replaced by gas and liquid chromatographic methods. The major limitations to thin layer chromatography were time, sensitivity, and difficulty in quantification. Gas chromatography presented a much better prospect. However, one problem encountered in gas chromatography was the fact that aldehydes are very reactive and must be derivatized prior to separation if high temperatures are to be used. With the development of liquid chromatography (LC), aldehyde analysis was much improved. One such LC method involved the use of pre-column derivatization by reaction of carbonyl compounds with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone derivatives (2,4-DNPH-derivatives). The first to employ this method were Papa and Turner (18). In their study, they examined separation of carbonyl compounds using 2,4-DNPH derivatization with gas chromatography and liquid chromatography. They found that liquid chromatography offered a definite advantage: the separations were done at ambient temperatures, rather than at the high temperatures required to vaporize the aldehydes for GC analysis, which resulted in no component decomposition. There were some disadvantages. This method worked well for less polar aldehydes, but resulted in long retention times (35 min.) for more polar aldehydes such as formaldehyde. Another disadvantage was the use of a gradient mobile phase composed of less desirable solvents such as hexane and chloroform.

Since aldehydes as a class are relatively polar compounds, reversed phase chromatography was studied by Selim in 1977 (19). In this study, the separations of

aldehydes and ketones were compared when using normal phase (adsorption) chromatography and reversed phase (absorption) chromatography. In the reversed phase study, a μBondapack C_{18} column was used, with an acetonitrile-water mobile phase followed by UV detection at 336 nm (19). Some of the disadvantages of this method included the preparation of the 2,4-DNPH-aldehyde derivative and the need for recrystallization followed by three solvent extractions, which was very time-consuming. However, this was still an improvement over GC analysis because the method could be used to analyze high molecular weight or thermally unstable compounds. One valuable bit of information was also gained from this experiment. It involved the use of the propionaldehyde-DNPH derivative on a microscale at room temperature. According to Selim, an equilibrium was established in the formation of the 2,4-DNPH-propionaldehyde derivative (19). See the equilibrium reaction in Figure 6.

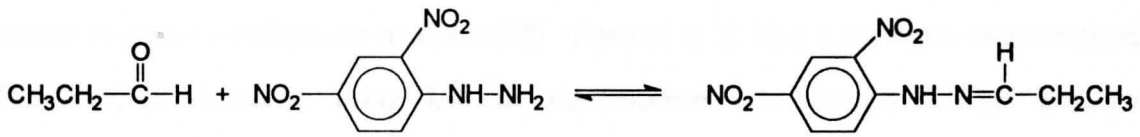


Figure 6: Propionaldehyde-DNPH Equilibrium Reaction. Propionaldehyde reacts with 2,4-dinitrophenylhydrazine to form propionaldehyde-dinitrophenylhydrazone.

In gram scale studies of the derivatization of propionaldehyde, it was found that it precipitated out immediately due to its low solubility in the aqueous phase. When the derivative was removed from the aqueous phase by precipitation, the equilibrium was shifted toward the formation of more derivative. Thus the reaction was almost quantitative. Selim concluded, "...the water insolubility of propionaldehyde indicates that the reaction could be made quantitative by using an aqueous-organic two-phase reaction medium" (19). Propionaldehyde reacted with 2,4-DNPH in the aqueous phase and partitioned into the organic phase, thus allowing the reaction to go to completion. Selim

also reported some advantages of reversed phase chromatography over adsorption chromatography in the separation of non-polar derivatives (19).

Another team of researchers working for the Reynolds Tobacco Company, Mansfield, et al., applied the 2,4-DNPH method to the detection of formaldehyde in cigarette smoke, using reversed phase chromatography, and a mobile phase of iso-octane, methylene chloride, acetonitrile, and methanol (20). They used a μPorasil silica column, and reported detection of formaldehyde in a similar range as those using a colorimetric method (21).

In 1989, three researchers, Benassi, Sememzato, and Bettero, developed a method for detection of free formaldehyde in cosmetics using 2,4-DNPH derivatization and reversed phase chromatography (22). This method replaced one that used headspace diffusion and direct reaction with 2,4-DNPH followed by chromatography developed by Lam and Margiasso (21). One draw-back of the Lam-Margiasso method was that it took several hours to complete the analysis (22). Benassi et al. also considered other methods that involved detection using UV or fluorescence spectroscopy, HPLC and gas chromatography after derivatization with different reagents, but found that the sample pre-treatments were not suitable for routine control and stability studies (22). They sought to develop a "...rapid and reliable method based on direct reversed-phase HPLC of untreated samples after precolumn derivatization with 2,4-DNPH, using the standard additions method" (22). They used another approach to form the 2,4-DNPH-aldehyde derivative by combining the sample dissolved in THF-water, or acetonitrile-water, with acidified 2,4-DNPH, and stabilizing it by adding phosphate buffer and sodium hydroxide. By allowing the reaction to occur in the aqueous-organic solution that would be used for the chromatographic separation, the pretreatment and extraction steps were eliminated. The entire derivatization process took about three minutes, as opposed to previous extraction methods that took up to several hours. In their study, Benassi et al. also determined the stability of the 2,4-DNPH derivative. The product reached a maximum of formation

within 3 minutes, and afterwards, its concentration decreased as a function of time because of the derivative's instability in the acidic medium. To overcome this, a phosphate buffer was added and the solution neutralized with sodium hydroxide, which stabilized the reaction over a period of 60 minutes. The reaction reached a maximum yield after 3 minutes and was reproducible.

Using a method similar to that of Benassi et al., Houlgate et al. (16) applied the use of 2,4-DNPH to determine formaldehyde and acetaldehyde in cigarette smoke. They used precolumn derivatization followed by reversed phase separation using acetonitrile-water (1:1) as the mobile phase. They determined that the aldehyde-2,4-DNPH solutions were stable for at least eight days when stored in a refrigerator. They also suggested flushing out the sample loop with the sample solution several times before introducing the sample into the system, followed by repeated flushing with acetonitrile after it was done.

An enhancement of the 2,4-DNPH method was applied to the analysis of aldehydes in air by using a Sep-Pak DNPH-silca cartridge followed by reversed-phase chromatography (14). This was a DNPH coated silica contained in a radially-compressed cartridge. They used water/acetonitrile/tetrahydrofuran mobile phase followed by UV detection. They reported detection of aldehydes to 1 ppb for a 1000 liter sample of air (14).

On-Line Reduction Reactors

Chemical Bed Reactor

As discussed earlier, chemical reduction can significantly alter the detectability of a compound. One reduction of interest is the reduction of nitro-polycyclic aromatic hydrocarbons (PAH) to amino polycyclic aromatic hydrocarbons (APAH) by the use of a zinc bed reactor. One such study was conducted by Sigvardson and Birks (23) in 1984. The reduction column was packed with a 1:1 mixture of glass beads and zinc dust of

similar particle size, and placed after the analytical column. The percent reduction was monitored by the appearance of the amino-PAH peak, or the disappearance of the nitro-PAH peak. The column had to be repacked daily, to minimize band broadening caused by changes in the zinc located at the head of the column. Band broadening was caused by a decrease in the particle size and the subsequent creation of voids at the heads of the columns. The glass beads extended the time that band broadening could be considered negligible. For the reaction, the eluent pH had to be about 6.5.

Electrochemical Reactor

A post-column electrochemical reactor was designed by Mike, et al. (24) in 1990. The two-electrode system used common and readily available "off the shelf hardware" and was used to oxidize phenlypropanolamine for enhancement of HPLC-UV detection (24). A drawback of the system was that the electrode needed to be repacked every 2 to 3 days of use. Although the use of the two electrode cell was very simple, there was no way to reliably control the oxidation potential. Several points of interest came out of this work. The stainless steel fittings used were inert--there was no material build up on them. There was a greater yield of the product when the working electrode was placed before the auxiliary electrode. The reactor itself contributed very little to overall band broadening. And lastly, the concentration of the supporting electrolyte could be determined (24).

Another related study by Ramos and Mike (8) utilized the electrochemical reactor for fluorescence detection of catecholemines. High oxidation potentials were involved, and the system was shielded by a rubber tube. Advantages over direct electrochemical detection include a lack of electrode poisoning problems, and decreased sensitivity to variations in temperature and flow rate, and to mobile phase impurities.

Aldehyde Separation via HPLC and Fluorescence Detection.

There are several methods that utilize direct reaction of an aldehyde with a fluorogenic reagent followed by chromatographic separation and fluorescence detection. One early method used reaction of aldehydes with 2-diphenyl-acetyl-1,3-indandione-1-hydrazone, followed by separation on paper chromatography (25). The detection limits were near 2 ng per spot. No studies were conducted using this reagent and HPLC.

One of the first methods to employ fluorescence derivatization and chromatography in the analysis of aldehydes involved derivatization with dansyl hydrazone (26). Although it produced detection limits of 1 - 10 ng/injection, it had several drawbacks: a lengthy preparation time of the derivative, the need for evaporation of the reaction solvent, and the need to dissolve the product in a suitable chromatographic solvent prior to injection.

Another method was developed for the separation, identification and quantification of aldehyde and ketone mixtures by Uzu, et al. (27) in 1990. It involved pre-column derivatization with fluorogenic reagents, separation via HPLC, and fluorescence emission detection. They reported detection limits in the sub-pmol to pmol range. There were several advantages to this method, including: non-fluorogenic reaction by-products were generated that would not interfere with detection, and high selectivity for aldehydes and ketones. Again there were numerous drawbacks to this method. Most importantly, the fluorogenic reagents were not readily available and needed to be synthesized, and the derivatization process was very lengthy.

Another study of aldehyde/ketone-specific fluorogenic reagents was conducted by Traore, et al. (28). Luminarin 3, Luminarin 11, and Luminarin 13, were reacted with aldehydes and ketones at room temperature and separated via HPLC and fluorescence detection. Detection limits were in the sub pmol range. Again, time of reaction was a major drawback, with reaction times varying from 30 - 120 minutes.

A method developed in Japan, by Iwata, et al. (29), used selective reagents that reacted with long chain aldehydes and aromatic aldehydes. This method gave detection limits in the fmol range. Limitations of this method were the reaction time of 1 hour for heptanal, and the difficulty in obtaining the chemical, which was manufactured in Japan.

Fluorescamine Studies

4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione, commonly known as fluorescamine, and commercially as Fluram, was synthesized in 1972 by Weigele, et al. (30). The spirolactone was used to supersede the fluorgenic ninhydrin reaction used in peptide analysis. It reacted with the primary amino group in the peptide and formed a fluorgenic product (30), with excitation and emission maxima at 390 nm and 475 nm respectively (31). The reaction occurred best at pH 8-9 with an excess of reagent in a water-miscible non-hydroxylic solvent, such as acetone or acetonitrile at room temperature (30). The reagent was non-fluorogenic itself, and excess reagent was concomitantly destroyed with a half time of several seconds (32). The hydrolysis products are non-fluorescent (33). The reactions are shown below:

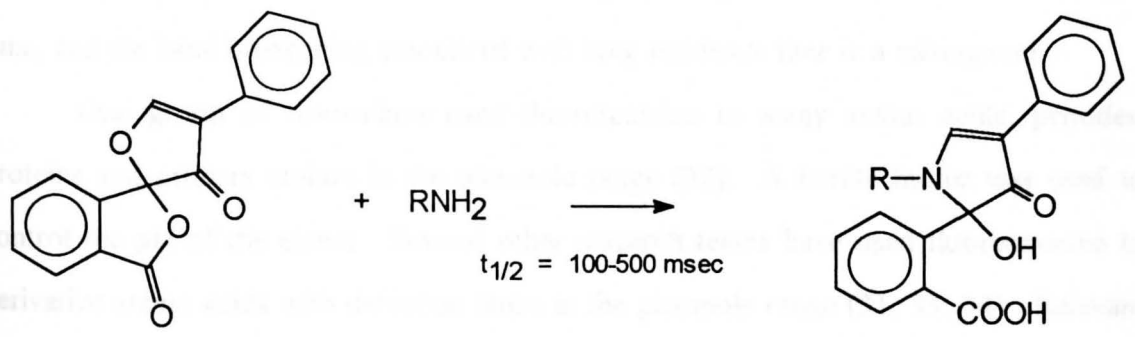


Figure 7: Fluorescamine Reaction. The reaction of Fluorescamine with a primary amine at pH 9. The half-life of the reaction is shown (33).

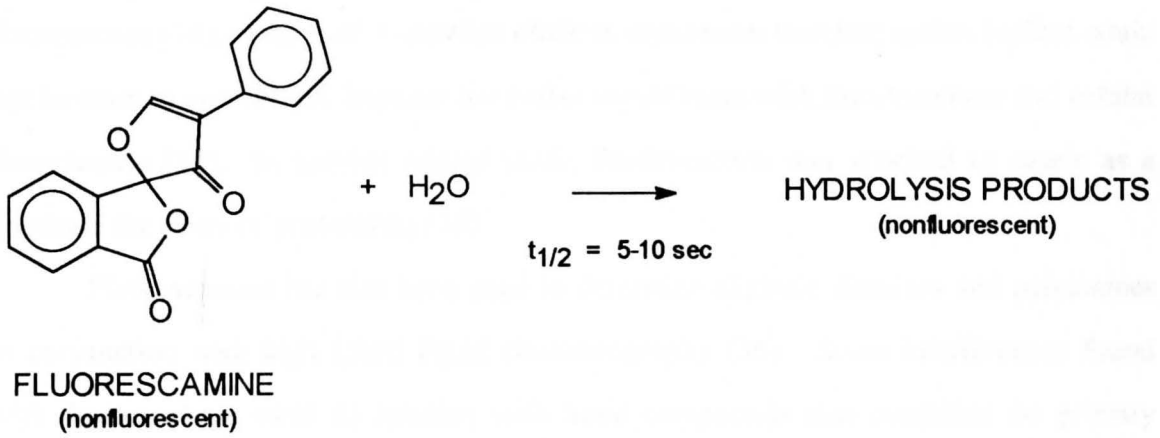


Figure 8: Fluorescamine Hydrolysis Products. Reaction of Fluorescamine with water at pH 9. The half-life of the reaction is shown (33).

Weigele et. al. reported detection limits down to the nanomole/mL range, and determined that the reaction proceeds to 90% completion in aqueous solutions (30). The fluorescence was proportional to the amine concentration and the fluorophors were shown to be stable for over several hours (32). Free ammonia did not interfere (32). Another compound that was commonly reacted with primary amines to form fluorophors was o-phthalaldehyde. However, five minutes of mixing in a reaction coil were required before detection was possible (25). The obvious draw-backs of using this reagent were the increased retention time, and the band broadening associated with long residence time in a mixing coil.

One group of researchers used fluorescamine to assay amino acids, peptides, proteins and primary amines in the picomole range (32). A borate buffer was used to control the pH of the eluent. Several other research teams have used fluorescamine to derivatize amino acids with detection limits in the picomole range (31, 33, 34). Relevant conclusions from these studies were: the fluorescamine reaction time was fast (33) and in reaction with amino acids, stability was obtained after five minutes (34). However, researchers were not certain if all amino groups had reacted completely, and gave no reason to expect all fluorescamine-substituted amino groups to exhibit the same quantum

fluorescence (34). One final somewhat obvious conclusion was that amino buffers could not be used to control pH, because the buffer would react with fluorescamine and exhibit fluorescence (34). In another related study, fluorescamine was attached to casein as a substrate for assay of proteinases (35).

Fluorescamine has also been used to determine aliphatic diamines and polyamines in conjunction with high speed liquid chromatography (36). Some interferences found with fluorescamine were its reaction with basic compounds that contained no primary amino group, but resulted in a pale yellow colored chromophore with no fluorescence behavior (36).

In a special study on post-column derivatization in HPLC, Frei, et al. investigated the reaction of fluorescamine with a nonapeptide in an attempt to determine the best reaction conditions without appreciable band broadening (37). They concluded the optimum reaction time was 50 seconds, with a decrease in the fluorogenic product of 5% after 5 minutes. The optimum conditions for minimizing band broadening at the reagent addition mixing tee and the reactor were using a short spiral of narrow diameter tubing, along with a mixing tee of such a geometry that the reagent enters at a 30 degree angle against the eluent stream. This allowed for enough turbulence and radial mixing to reduce the band broadening by 30%, as compared with the standard 90 degree mixing tee. Use of a bed reactor was also considered, and resulted in an increase in band broadening by 20%. They also determined that using acetonitrile as the reagent solution caused bubbling in the eluent upon mixing with water in the mobile phase due to the release of dissolved air.

CHAPTER III

STATEMENT OF PROBLEM

Some common difficulties encountered in High Performance Liquid Chromatographic analyses are inadequate detectability of analytes in the chromatographic eluent and overlapping bands resulting in chromatograms cluttered with irrelevant peaks. These problems make separation, identification and detection of aldehydes problematic. There is also an inherent problem in using UV detection: the requirement of the presence of a suitably strong chromophore in the analyte, that must absorb within a useful range of the detector (24). Not only does the chromatogram show other compounds, but UV detection does not produce detection limits as sensitive as other methods such as fluorimetry.

As noted earlier, as a class, aldehydes are very reactive. However aldehydes react directly with few compounds that yield fluorescent derivatives. Some of these compounds are hard to obtain from common chemical suppliers, and/or they involve lengthy time-consuming methods. A fast, easy-to-use, inexpensive method that exhibited both selectivity and sensitivity was desired. By taking advantage of the reactivity of aldehydes, a multistep derivatization method was proposed that would result in a fluorescent aldehyde derivative that could be separated from other aldehydes and other compounds in a matrix via high performance liquid chromatography (HPLC) with fluorometric detection. The proposed method would be relatively inexpensive, because it used readily available chemicals and was fast.

The main purpose of this study was to develop a chromatographic method in which a post column reactor could be utilized in the formation of fluorescent aldehyde derivatives to improve the selectivity and sensitivity of aldehyde detection. The

electrochemical reactor would allow for careful control of the multistep reduction reaction of the nitro groups of 2,4-dinitrophenylhydrazone-aldehyde derivatives to diamino groups, forming 2,4-diaminophenylhydrazone-aldehyde derivatives (2,4-DAPH-aldehyde derivatives). This primary amino group-containing derivative compound could then be derivatized with fluorescamine, and it was proposed that a fluorescent compound would result, which could then be detected by fluorimetry. Thus detection limits of the aldehydes could be improved over UV detection limits.

The advantage of using a post-column fluorgenic reactor was that it would remove the non-derivatizable compounds from detection, and thus give a simplified chromatogram with fewer peaks. This would make identification of the analytes of interest much less complicated, because there would be fewer peaks and reduced peak overlap caused from the interference of compounds not of interest. The usefulness of doing an on-line electrosynthesis was the elimination of manual chemical treatments to the sample. The post-column reduction also added an additional element of selectivity to the procedure. The compounds could be reacted with a fluorescing agent giving detection limits 10 to 1000 times lower than spectrophotometric detection. Inadequate detectability and specificity of the detector for a particular analyte or class of analytes may result in incorrect identification. Another advantage of using on-line electrosynthesis was the fact that the reaction could be controlled to improve the yield of the reduction products. It also made stability of the product unimportant and the method was fast.

There were several obstacles that had to be overcome in this project. One was the control of the reduction potential of the on-line electrochemical reactor. The reduction of nitro groups to amino groups involved a three step process. If the reduction was not complete, reaction with fluorescamine would not occur, because there were no free primary amino-groups. The two-electrode system used by Ramos and Mike allowed for no definite control of the electrode potential. It was proposed that this problem would be overcome by the development of a flow-through three-electrode system for HPLC.

Another problem was the reaction of a fluorogenic reagent with 2,4-DAPH-aldehyde derivatives, a large molecule with steric hindrance and a complex structure. The proper fluorogenic reagent must react to give fluorescent derivatives and adequate mixing must occur, without extensive band broadening.

CHAPTER IV

MATERIALS AND APPARATUS

Chemicals

All reagents were of analytical grade, or of the highest grade available. Table 1 summarizes the chemicals that were used, and their manufacturers.

Table 1: List of chemicals used and their manufacturers.

Chemical	Manufacturer
Propionaldehyde	J. T. Baker (Phillipsburgh, NJ)
Potassium dihydrogen phosphate	"
Boric acid, acetonitrile (HPLC grade)	"
Acetone (HPLC grade)	"
2,4-dinitrophenylhydrazine (70%)	Aldrich (Milwaukee, WI)
Zinc dust (325 mesh and 100 mesh)	"
Platinum powder	"
Silver wire (0.5 mm diameter)	"
Acetaldehyde	Eastman (Rochester, NY)
Butyraldehyde	"
Valeraldehyde	"
Formaldehyde (37%)	Sigma (St. Louis, MO)
Fluorescamine	"
Concentrated nitric acid (69.0-71.0%)	VWR Scientific (Norwood, OH)
Concentrated hydrochloric acid (36.5-38.0%)	"
Disodium hydrogen phosphate	Fisher Scientific (Fairlawn, NJ)
Sodium hydroxide	EM Science (Gibbstown, NJ)
Sodium perchlorate monohydrate	G. F. Smith Co. (Columbus, OH)
Reference Electrode filling solution	Orion Research (Cambridge, MA)

Chromatographic materials and instrumentation

Chromatographic separations and analyses were carried out using an Econosphere C-18, 5μ reversed phase column (250 mm, I.D. 4.6 mm) purchased from Alltech

(Deerfield, IL), as were all connectors, mixing tees and tubing. Model 110 B Pumps (Beckman Instruments, Fullerton, CA) were used to deliver the mobile. Helium was used to degas the mobile phase prior to introduction into the system. A Model 406 analog interface (Beckman) was used to interface the chromatography instrumentation with the Beckman System Gold chromatography computer interface program. For all HPLC/UV measurements, A Beckman Model 168 diode array detector was used. HPLC/fluorescence measurements were done using a Model 2001A Fluoro-Tec fluorescence detector (St. John's Associates, Beltsville, MD) equipped with excitation and emission filters with wavelength maxima at 254 to 420 nm and 450 nm cutoff, respectively. An adjustable, piston driven lab pump, Model QSY, and pulse dampener (Fluid Metering Incorporated, Oyster Bay, NY) was used to introduce the post-column derivatizing reagent in to the system.

Other Instrumentation

UV measurements of bulk solution standards were done on a Diode Array Spectrophotometer, Model 8452A, purchased from Hewlett Packard (Wilmington, DE). All bulk solution spectrofluorometric measurements were conducted on a Spectrofluorophotometer, model RF 5000U, purchased from the Shimadzu Corporation, Kyoto, Japan.

On-Line Reduction Reactors

Zinc Column

A zinc reduction column was fabricated from a piece of stainless steel tubing, 2.00 mm I.D., 2.00 mm thick, and 5.0 cm long. The column was filled with zinc dust, 325 mesh, and assembled as shown in Figure 9. The column was connected to a solvent pump and 55% acetonitrile-water was pumped though at a rate of 1.5 mL/min. The column head was removed and more zinc dust added until the column was filled. It was

connected back up to the solvent pump and flushed with solvent and the process of re-packing was repeated until there was no more head-space at the inlet of the column.

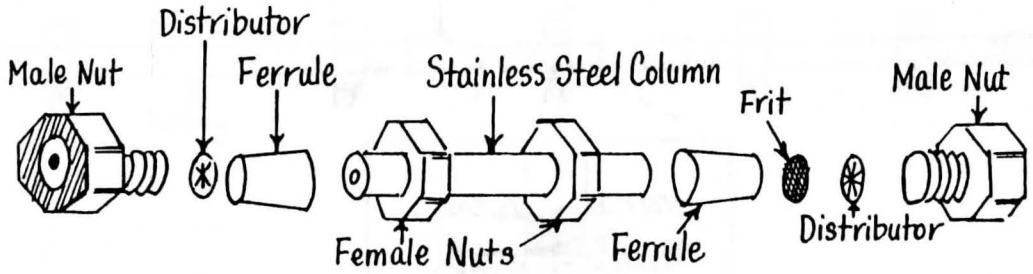
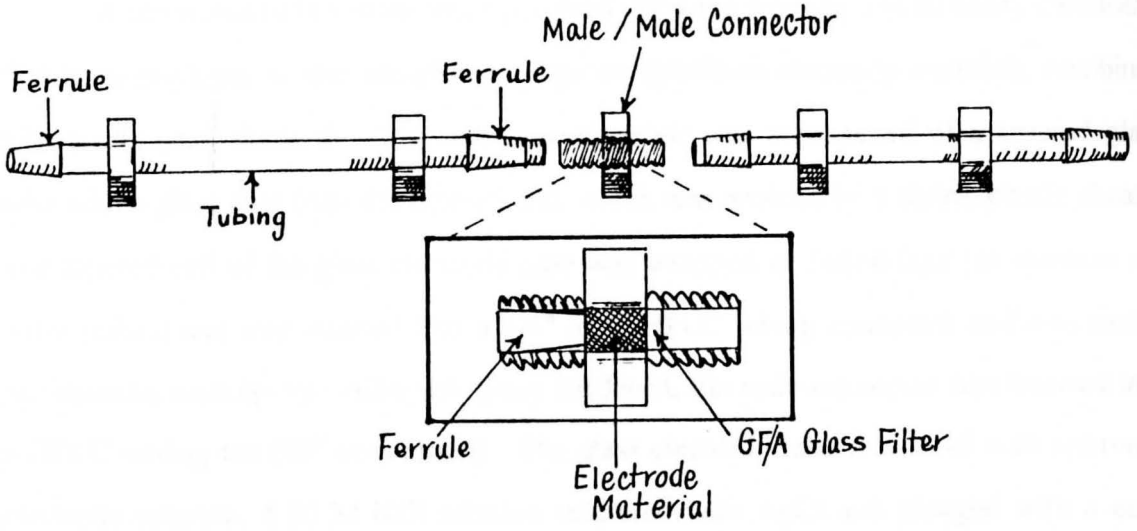


Figure 9: Zinc Bed Reactor Design and Construction.

Electrochemical Reactor: Two-electrode System

A two-electrode on-line reactor was assembled according to the procedure given by Mike et al. (24), and Ramos and Mike (8), as shown in Figure 10. The reactor was assembled from two 1/16-in. stainless steel male/male connectors, which were joined by a 0.90 cm length of 1/16-in. (0.01 in. I.D.) Flexon tubing. The working electrode (cathode) was packed with either platinum powder or zinc dust (325 mesh), and placed in series before the unpacked auxiliary electrode (anode). The electrode material was kept in the cathode with a 1/16 in diameter stainless steel frit. Electric potential was supplied for the two-electrode system by an electrophoresis-type power supply Heath Schlumberger, model SP-17A, (Heath, St. Joseph, MI). This power supply was capable of providing a potential from 0 to 400 volts, at a current up to 150 mA (16). The voltage and current range of the power supply presented the danger of electric shock, so the reactor was shielded by a radially split piece of 1-in. I.D. rubber tubing which covered the reactor assembly.

(a)



(b)

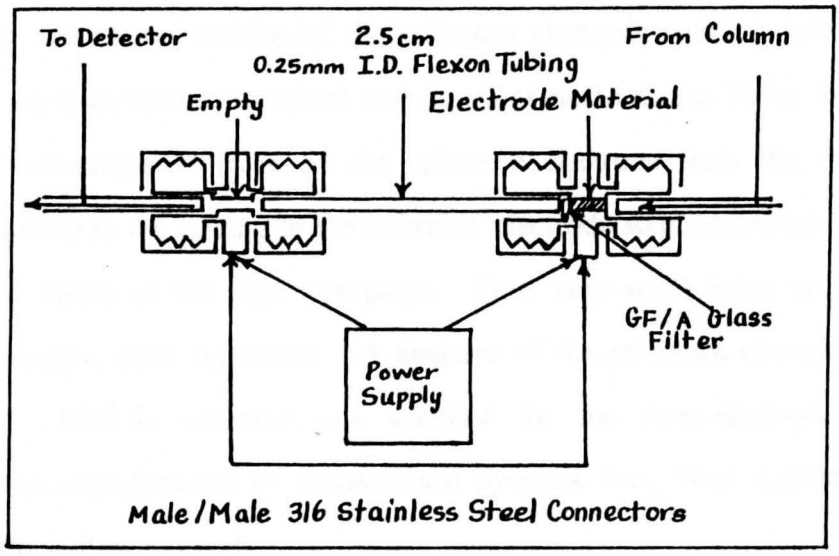


Figure 10: Two-Electrode Flow-Through Reactor Design. (a) Assembly of the working electrode. (b) Cross-sectional view of the working and auxiliary electrodes.

Three-Electrode System.

A three electrode system was assembled using the working and auxiliary electrodes that were employed in the two-electrode system (platinum electrode material), combined with a reference electrode. The reference electrode was constructed of a tapered glass tube with a glass plug over the tapered end, which was retained by a shrink-plastic sheath. The tapered end of the glass electrode tube was wrapped in Teflon tape (to increase the outer radius) and was inserted into a 1/4" male HPLC tubing connector and was sealed permanently with epoxy. After the epoxy hardened, the male connector was inserted into a HPLC mixing tee (90^{o} orientation). The glass electrode tube was filled with reference electrode solution, 4.00 M KCl solution saturated with AgCl and plugged with a cork lanced vertically with a silver wire, 0.5 mm diameter. The silver wire was connected to a lead and the top of the tube was sealed using a rubber sleeve. The reference electrode was sealed at all junctions with epoxy and connected to the chromatographic eluent stream. Small holes that caused leaking of the reference electrode solution were plugged with epoxy and the electrode was wrapped with layers of electrical and Teflon tape. There was excessive back-pressure placed on the reference electrode from the chromatographic system, resulting in the forcing of the reference electrode solution though any opening or lapse in the layers of the tape wrappings. Thus very small holes or crevices in the wrapping became quite significant. A diagram of the reference electrode is shown in Figure 11. Electric potential was supplied for the three-electrode system by a potentiometer manufactured by Bioanalytical Systems, Inc., West Lafayette, IN, model CV-27 cyclic voltammograph.

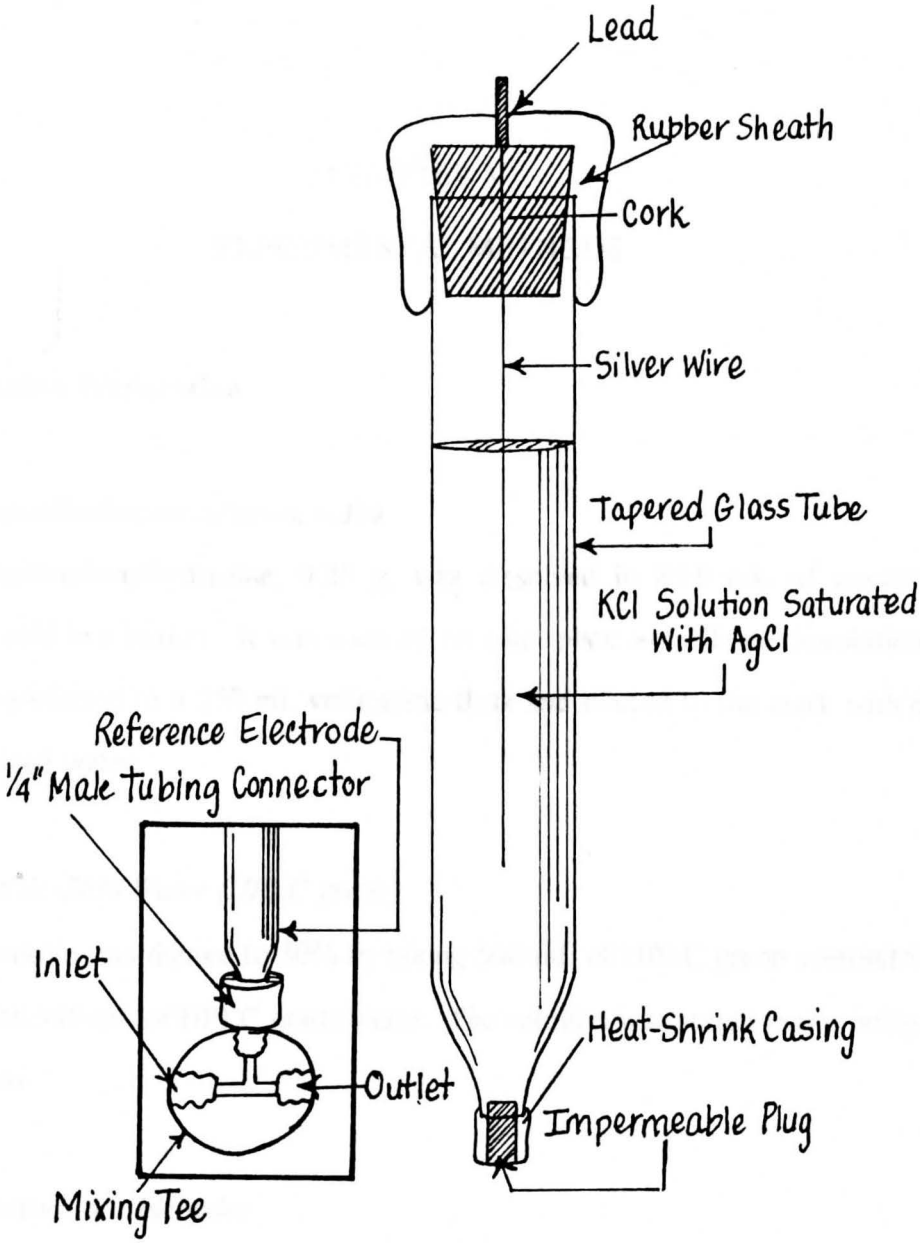


Figure 11: Three-Electrode Reference Electrode Design. The design of the reference electrode is shown in cross-section. The inset shows the mounting of the reference electrode into the mixing tee for electrical contact with the chromatographic eluent.

CHAPTER V

EXPERIMENTAL METHODS

General Solution Preparation

2,4-dinitrophenylhydrazine solution, 0.1%

2,4-dinitrophenylhydrazine, 0.25 g, was dissolved in 85.9 mL of concentrated hydrochloric acid in a beaker. It was warmed on a hot plate to facilitate dissolution, then cooled and transferred to a 250 mL volumetric flask and diluted to the mark with double filtered deionized water.

50% Acetonitrile-50% Water (HPLC grade)

Acetonitrile was diluted to 50% by taking 500 mL of HPLC grade acetonitrile and combining with 500 mL of HPLC grade water. The solution was stored in the refrigerator when not in use.

Standard Solutions of Aldehydes

The primary solution of aldehydes was prepared such that the molar concentration of the aldehyde or mixture of aldehydes was 6.17×10^{-2} M. From this primary solution, successive dilutions were made to obtain the desired aldehyde concentrations. The 6.17×10^{-2} solutions of the individual aldehydes were prepared by taking 0.500 mL of 37.1% formaldehyde, 0.346 mL of acetaldehyde, 0.444 mL of propionaldehyde, 0.556 mL of butyraldehyde, 0.656 mL of valeraldehyde, and adding to a 100 mL volumetric flask partially filled with chilled 50% acetonitrile-50% water HPLC grade solution. The

contents of flask were diluted to the mark with 50% acetonitrile-50% water. From this solution, 1.000 mL was taken by electronic pipette and diluted to 100 mL in a volumetric flask with 50% acetonitrile-50% water, resulting in a 6.17×10^{-4} M solution of each aldehyde. A solution of 6.17×10^{-6} M concentration was prepared by taking 1.000 mL of the 6.17×10^{-4} M solution and diluting to 100 mL in a volumetric flask with 50% acetonitrile. Solutions of different aldehyde concentrations were prepared by making similar dilutions. These solutions were stored in the refrigerator and were discarded and reprepared weekly (23).

Fluorescamine, 0.050%

Fluorescamine reagent was prepared by dissolving 0.0500 g of fluorescamine in enough acetonitrile or acetone to make 100 mL in a volumetric flask.

Mobile Phase with Supporting Electrolyte

A 0.1 M sodium perchlorate solution in 55% acetonitrile-45% water was prepared by combining 550 mL of HPLC grade acetonitrile with 450 mL of double-filtered deionized water and mixing. Sodium perchlorate monohydrate, 14.054 g, was dissolved in 1000 mL of the 55% acetonitrile-45% water solution in a 1000 mL volumetric flask.

2,4-dinitrophenylhydrazone Derivatization Procedure

Derivatives were prepared by placing 1.000 mL of the standard aldehyde solution, or sample, in a 80 mm x 750 mm test tube, and adding 0.400 mL of the 0.1% 2,4-dinitrophenylhydrazine solution, stoppering it, and mixing on a vortex mixer for 60 seconds. After a 2 min. reaction period, 0.400 mL of the 0.1 M phosphate buffer was added, followed by 0.700 mL of the 1.00 M sodium hydroxide solution. The test tube was corked again and the contents mixed for thirty seconds on the vortex mixer. A blank

derivative was prepared in exactly the same manner, only 1.000 mL of 50% acetonitrile-50% water (HPLC grade) was used instead of 1.000 mL of the standard aldehyde.

Chromatographic Studies

Separation of 2,4-DNPH aldehydes HPLC/UV

The method of separation and analysis was based on the method developed by Benassi et al. for the determination of formaldehyde in cosmetics (7). The mobile phase used was acetonitrile-water. The sample size was 10 μL . The derivatives were separated on a reverse phase column, with flow rate at 1.000 mL/min. Detection was at dual wavelengths, 254 nm and 345 nm. Aldehyde-2,4-DNPH derivatives were identified by appearance on chromatograms at both wavelengths.

Determination of Mobile Phase Composition

The composition of the mobile phase was determined by doing multiple separations of a 6.17×10^{-4} M mixture of aldehydes. A solvent gradient started at 5% acetonitrile-95% water, and increased to 100 % acetonitrile over a period of 30 minutes. All factors were held constant, except for the concentration of acetonitrile in the mobile phase. From the resulting chromatogram with the gradient superimposed over it, a concentration range was determined. Then using gradient programming, the mobile phase was changed to 77.5%, 70 %, 67.5%, 60 %, 55 %, 50%, 30%, 40% of acetonitrile with the remaining percent HPLC grade water.

Determination of Retention Times

Retention times of aldehydes were found by preparing 6.17×10^{-4} M solutions of the individual aldehydes, making the derivatives, and injecting 10 μL of the derivative in 65% acetonitrile-35% water, and comparing the individual aldehyde chromatogram with

the blank. The retention time of each 2,4-DNPH-aldehyde derivative was determined by peak appearance at both wavelengths (254 and 345 nm) in the aldehyde chromatogram, with no corresponding peaks in the blank chromatogram. The retention time of each aldehyde was confirmed by plot of a logarithm of retention time versus carbon number of a homologous series plot.

Post column Zinc Reduction HPLC/UV

The zinc column was placed in the chromatography sequence after the analytical column and prior to the detector. A derivative blank, 10 μL, was introduced into the system after a steady baseline was obtained. The flow rate was 1.00 mL/min., the mobile phase 55% acetonitrile-45% water, and UV detection was done at 254 and 345 nm. Chromatograms were obtained for the derivative blank and the 6.17×10^{-4} M aldehyde mixture. Diode array scans were taken from 190 to 400 nm.

UV Spectra on HP diode array

The extent of the zinc reduction reaction needed to be determined. This was done by conducting a batch investigation of the 2,4-diaminophenylhydrazone derivative UV spectra. Two derivatives of each of the aldehydes were prepared as usual. To one of each of the 2,4-DNPH derivatives, an excess amount of zinc dust was added directly to the test tube and was mixed on the vortex mixer for 60 seconds. Immediately, the yellow-colored solution turned colorless and bubbles formed. The colorless solution was decanted and the UV spectrum was scanned and compared with the spectrum of the 2,4-DNPH-aldehyde. Wavelengths of strong absorbance of the 2,4-DAPH-aldehyde derivative were obtained. This determination was done using formaldehyde, acetaldehyde, and propionaldehyde separately.

Comparison of UV Spectra from Beckman Diode Array

Diode array scans of UV spectra as determined by the chromatography detector of the blank and aldehyde mixture chromatogram mentioned in the subsection above were examined at the peak crest of each of the aldehydes, and the unreacted 2,4-DNPH and the dead volume. These were compared with the diode array scans taken at the same peaks from the chromatogram obtained after the reduction column was installed. The wavelength of maximum absorbance was determined for each aldehyde component.

On-Line Derivatization with Fluorescamine (zinc column)

Fluorescence Spectra

Batch investigations of derivatization of 2,4-DAPH-aldehyde with fluorescamine were begun by preparing a 2,4-DNPH derivative in the manner listed previously, and reducing the derivative in bulk solution with excess zinc dust, mixing and transferring the liquid to a larger test tube. To this test tube, an excess amount of fluorescamine was added, followed by vortex mixing and decantation of the solution. The excitation and emission spectra were measured on the Shimadzu spectrofluorophotometer. The aldehydes used were formaldehyde, acetaldehyde and propionaldehyde. The aldehyde-fluorescamine derivatives were measured individually, and not in the form of an aldehyde mixture.

Determination of HPLC conditions

The HPLC system was modified to include a reagent pump and a mixing tee. The UV detector was removed, and replaced by a fluorescence detector. The scheme is shown in Figure 12. Fluorescamine, 0.050%, was pumped through the reagent pump at 1.00 mL/min. The 2,4-DNPH-aldehyde derivative was prepared in the usual manner and 10 μL was injected into the system. The flow rate of the mobile phase was 1.00 mL/min. unless

otherwise noted. Optimum conditions for the reaction of fluorescamine and the 2,4-DAPH-aldehyde were studied. Several variables were changed over many determinations. The flow rate of the mobile phase and the reagent were reduced to 0.70 mL/min. The mobile phase was changed to 50% acetonitrile-50% water and the flow-rate raised back to 1.00 mL/min. The zinc column was changed to 100 mesh particles using 50% acetonitrile-50% water and flow rate 1.00 mL/min. The solvent was changed to acetone for the fluorescamine reagent in order to minimize bubbling from the mixing of water in the mobile phase, and acetonitrile in the reagent. A piece of 2.0 foot long tubing was coiled and placed directly after the mixing tee prior to the detector, with the mobile phase changed to 65% acetonitrile-35% water and flow rate to 0.70 mL/min. The integrator was disconnected and replaced with a strip-chart recorder to test the response of the detector. The flow rate was changed to 0.90 mL/min. and the mobile phase 55% acetonitrile-45% water, and a 2.0 ft coil reactor tube.

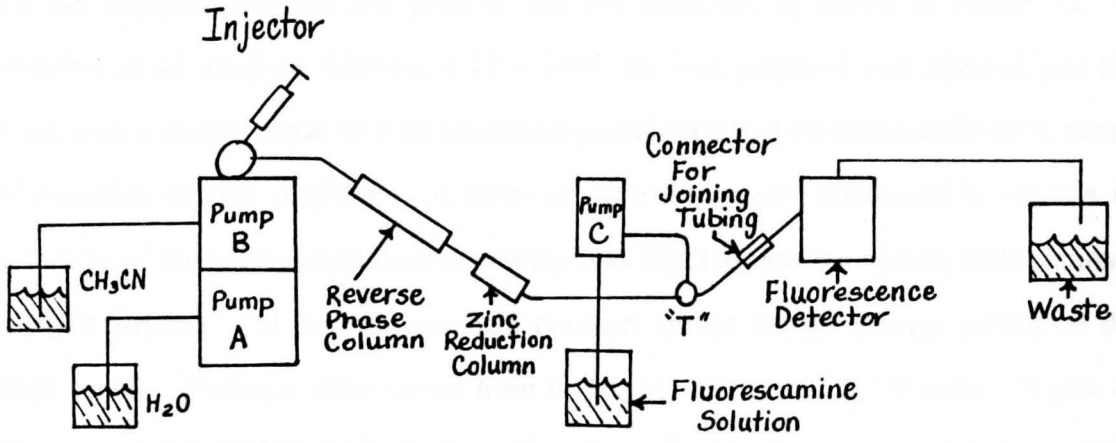


Figure 12: HPLC Configuration for Fluorescamine Studies.

Effects of Borate Buffer

A discrete study was conducted in which the derivative was monitored over time and emission measurements taken, for one sample with 1.000 mL of borate buffer (0.1 M), and the other with an equal volume of 50% acetonitrile-50% water. The excitation wavelength was set at 390 nm. Chromatographic application was performed by using 0.05% fluorescamine dissolved in acetone and pumping at a 2:1 flow ratio with borate buffer through the reagent pump.

Electrochemical Reactor

Electrochemcial studies were conducted first using a two-electrode system and then a three-electrode system and the results were compared.

Determination of potential/HPLC conditions--2 electrode system

The two-electrode system was installed in the chromatography system directly after the analytical column and prior to the UV detector, as shown in Figure 13. A derivative of an aldehyde mixture, 6.17×10^{-4} M, was prepared and injected into the system with a mobile phase of 0.10 M sodium perchlorate in 55% acetonitrile-45% water. UV detection was set at 212 nm. A series of experiments were conducted in which a 10 µL sample of the aldehyde mixture derivative was injected into the system with platinum electrode material. All factors were held constant except for the voltage setting on the power supply. Voltages were varied from 0, 10, 20, 25, 30, 40 to 50 volts. A plot of peak area of 2,4-DAPH-aldehyde derivative from the chromatograms and the voltage setting was made. The electrode was repacked with platinum powder and the series of experiments were repeated. The electrode packing was changed to zinc dust (325 mesh), and an aldehyde derivative was prepared (6.17×10^{-4} M). The mobile phase was the same as in the platinum study, and UV detection was at 345 nm and 212 nm. All other factors

were held constant again, except for the voltage setting on the power supply. Voltages were done in increments of 10 volts, starting with 10.0 volts and progressing to 70.0 volts.

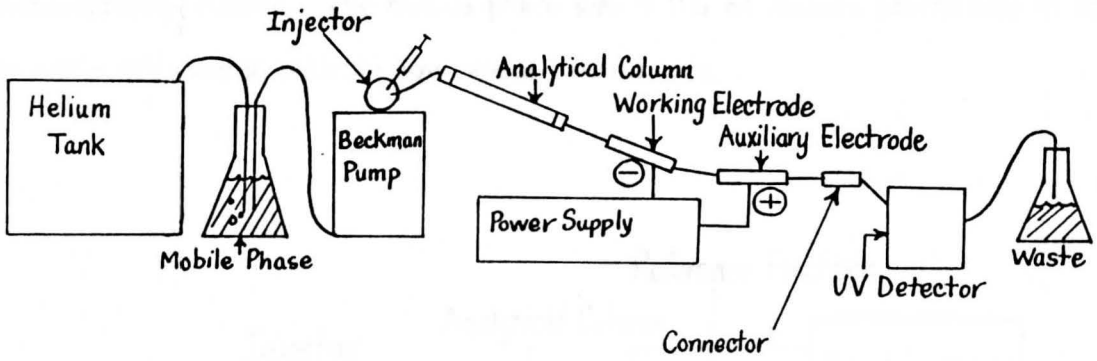


Figure 13: HPLC Configuration for Two-Electrode Studies.

Determination of Three-Electrode System--HPLC conditions

The reference electrode was connected to the chromatography system as shown in Figure 14. The potentiometer was set so that the potential difference on the working electrode was 0.10 volts. A 10.0 μL DNPH-blank sample was injected onto the chromatography column. The mobile phase was 0.100 M sodium perchlorate in 55% acetonitrile-45% water, with the flow rate at 1.00 mL/min.

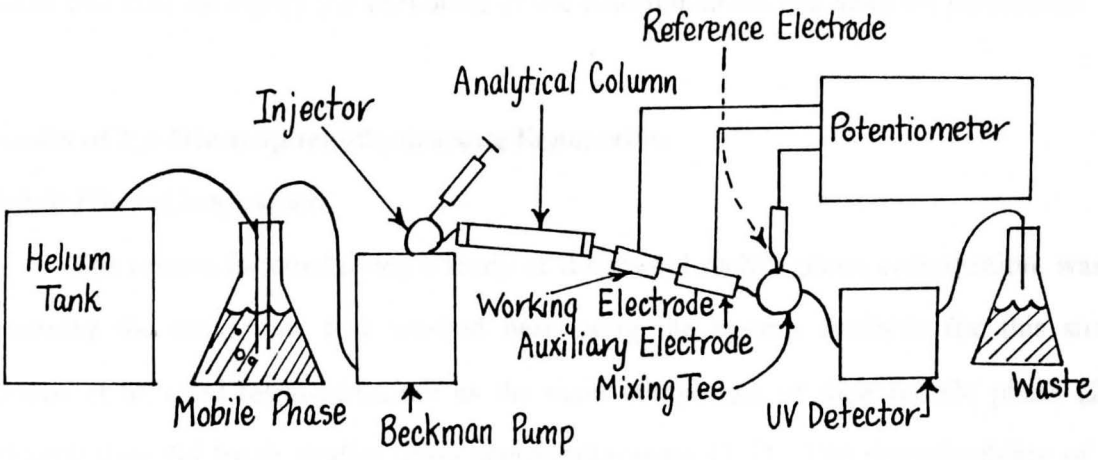


Figure 14: HPLC Configuration for Three-Electrode Studies.

CHAPTER VI

RESULTS & DISCUSSION

A number of experimental measurements and data were taken during each of the experiments outlined in the previous section. Presented in the following section are the results that best exemplify the outcomes of the determinations and analyses performed.

Results of 2,4-Dinitrophenylhydrazone Separation

Mobile Phase Composition

The reason for conducting a study of the proper mobile phase composition, was to determine the conditions that worked best using the system available for this study. Benassi et al. used tetrahydrofuran as the main component of their mobile phase (22), although they did batch studies using acetonitrile-water (1:1). The reproducibility of the derivatization method followed by reversed phase separation was also tested, although we used a mobile phase that was different from theirs.

Figure 15 shows a chromatogram that was obtained by using an acetonitrile solvent gradient from 5% acetonitrile to 100% acetonitrile over 30 minutes. The gradient was performed to allow "ball-park" chromatographic conditions to be selected in order to simplify separation optimization. The eluent was monitored by UV detection at 345 nm. This wavelength was chosen for presentation over the chromatogram monitored at 254 nm (as outlined in the literature) because the aldehyde peaks gave a higher absorbance at 345 nm, and there were less interfering compounds detected. In Figure 16, the optimum acetonitrile concentration as determined from the gradient run in Figure 15, 77.5% acetonitrile-22.5% water, is shown for the separation of the same mixture of aldehyde

derivatives. Figure 17 shows a chromatogram obtained when the mobile phase composition was changed to 50% acetonitrile-50% water. The optimum condition for the separation was finally determined to be 55% acetonitrile-45% water and is shown in Figure 18. This separation appeared to be better than the others because it provided good resolution of the chromatographic peaks, while minimizing the analysis time.

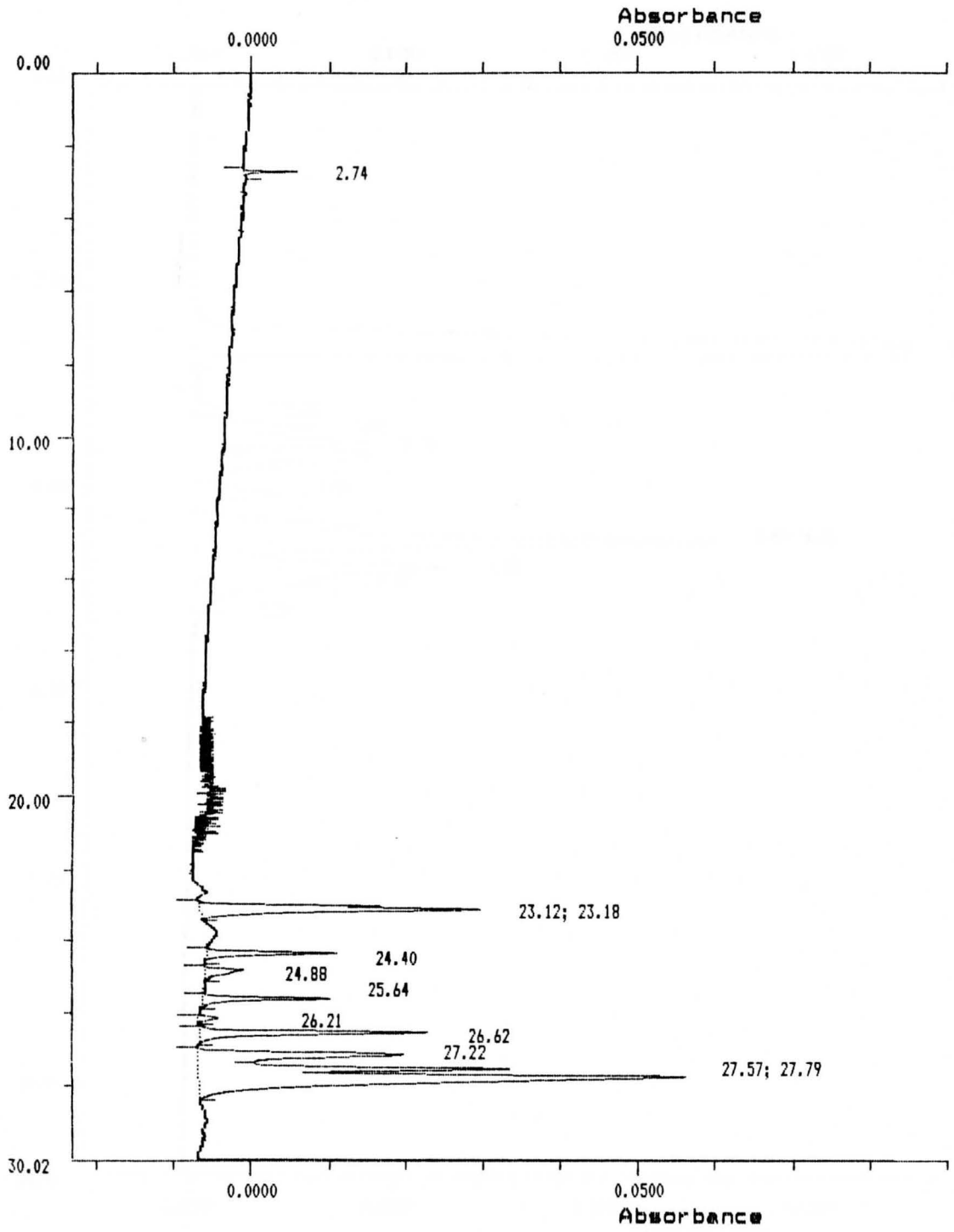


Figure 15: 2,4-DNPH-aldehyde mixture, 6.17×10^{-4} M.
Solvent gradient: 5 % acetonitrile-95% water to 100 % acetonitrile over 30 min. Flow rate: 1.00 mL/min. Ambient temperature. Sample size: 10 µL . UV detection, 345 nm.

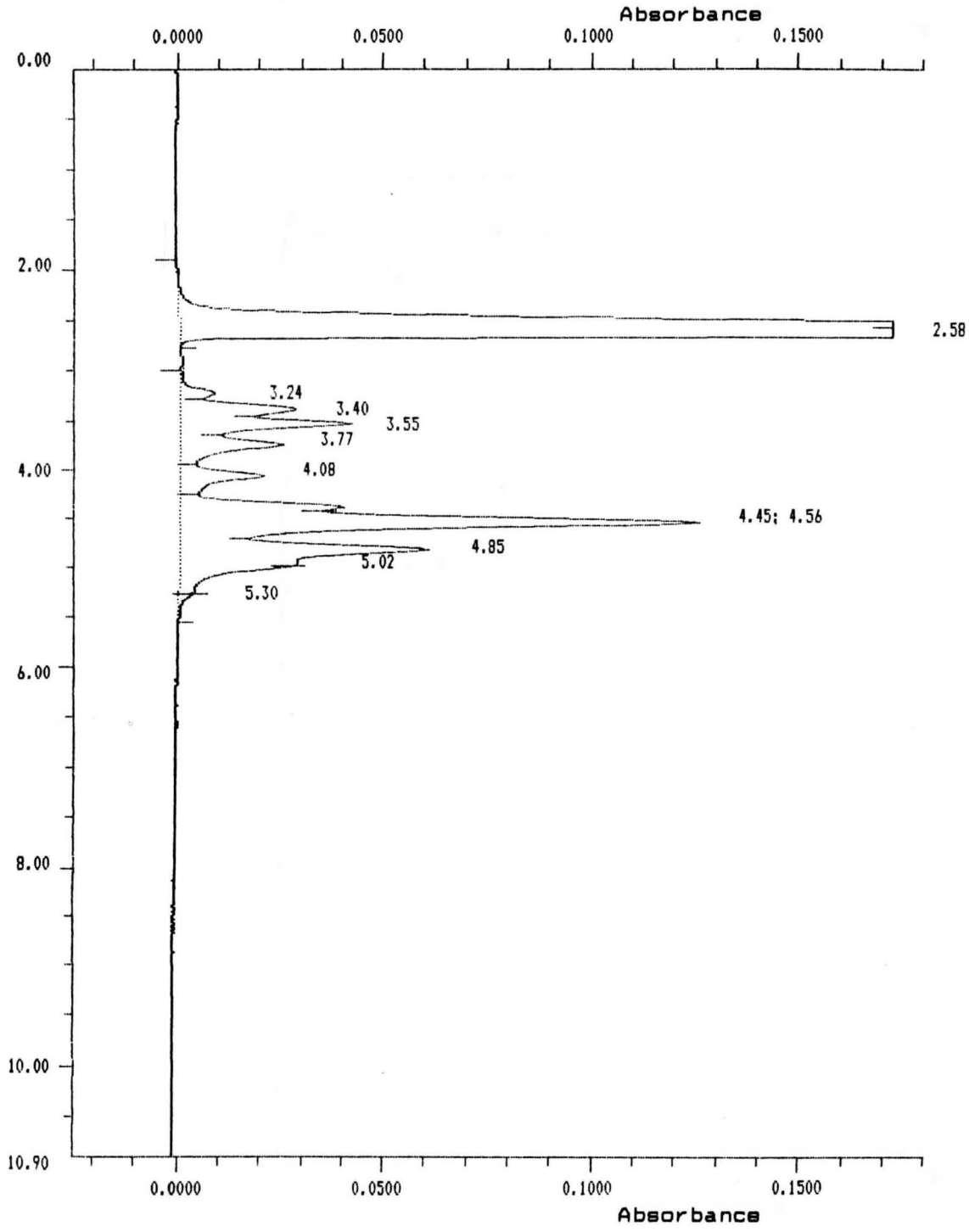


Figure 16: 2,4-DNPH-aldehyde mixture, 6.17×10^{-4} M. Mobile Phase: 77.5% acetonitrile-22.5% water. Flow rate 1.00 mL/min. Ambient Temperature. Sample size: 10 μL UV detection 345 nm.

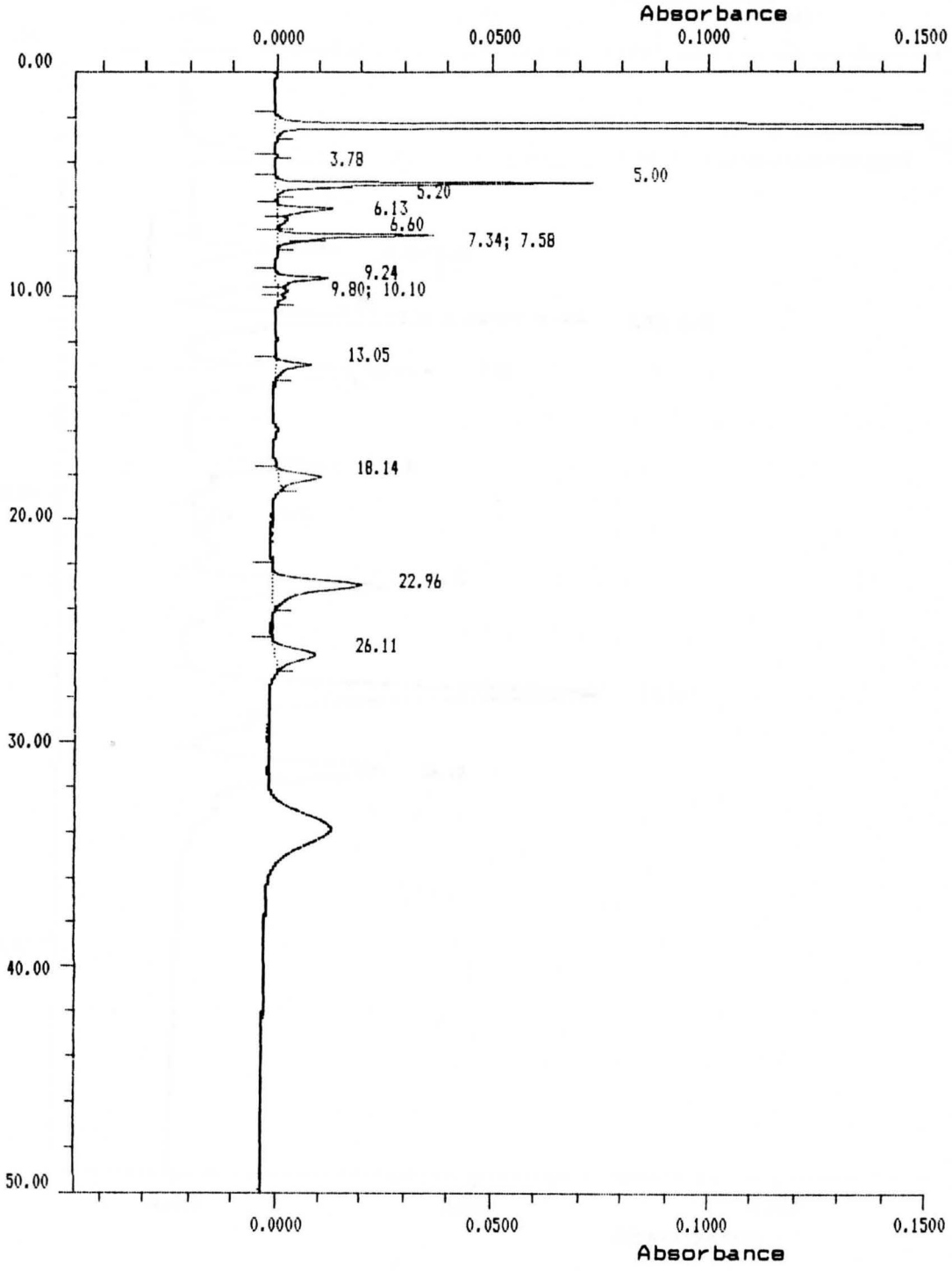


Figure 17: 2,4-DNPH-aldehyde mixture, 6.17×10^{-4} M. Mobile Phase: 50% acetonitrile-50% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 μL sample injection. UV detection 345 nm.

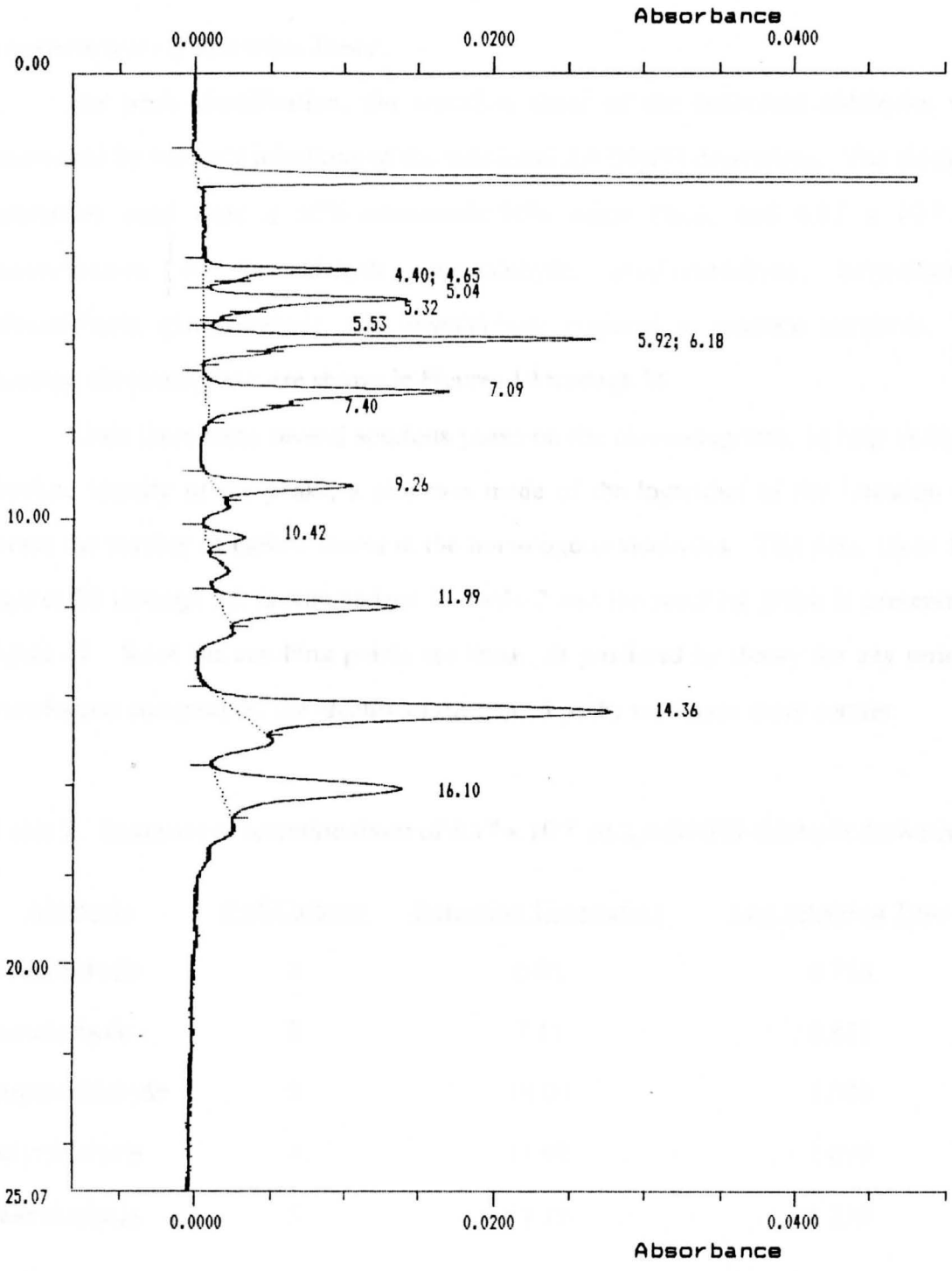


Figure 18: 2,4-DNPH-aldehyde mixture, 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow Rate: 1.00 mL/min. Ambient Temperature. Size: 10.0 μL. UV detection 345 nm.

Determination Of Retention Times

For peak identification, the retention times of the individual aldehydes were determined by separate injections of the individual 2,4-DNPH derivatives. The aldehyde-derivatives used were a 50% acetonitrile-50% water blank, and 6.17×10^{-4} M concentrations of formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, valeraldehyde, glutaraldehyde, and benzaldehyde prepared as separate standards. The resulting chromatograms are shown in Figures 19 through 26.

Since there were several spurious peaks on the chromatograms, to help verify the absolute identity of the peaks, a plot was made of the logarithm of the retention time versus the number of carbon atoms in the homologous aldehydes. This data, taken from Figures 20 through 24, is summarized in Table 2 and the resulting graph is presented in Figure 27. Since the resulting points are linear, as predicted by theory for any series of homologous compounds, the identity of the named peaks was made more certain.

Table 2: Summary of retention times of 6.17×10^{-4} M 2,4-DNPH-aldehyde derivatives.

Aldehyde	# of Carbons	Retention Time (min.)	Log retention Time
Formaldehyde	1	6.03	0.780
Acetaldehyde	2	7.21	0.858
Propionaldehyde	3	10.00	1.000
Butyraldehyde	4	11.99	1.079
Valeraldehyde	5	17.32	1.239

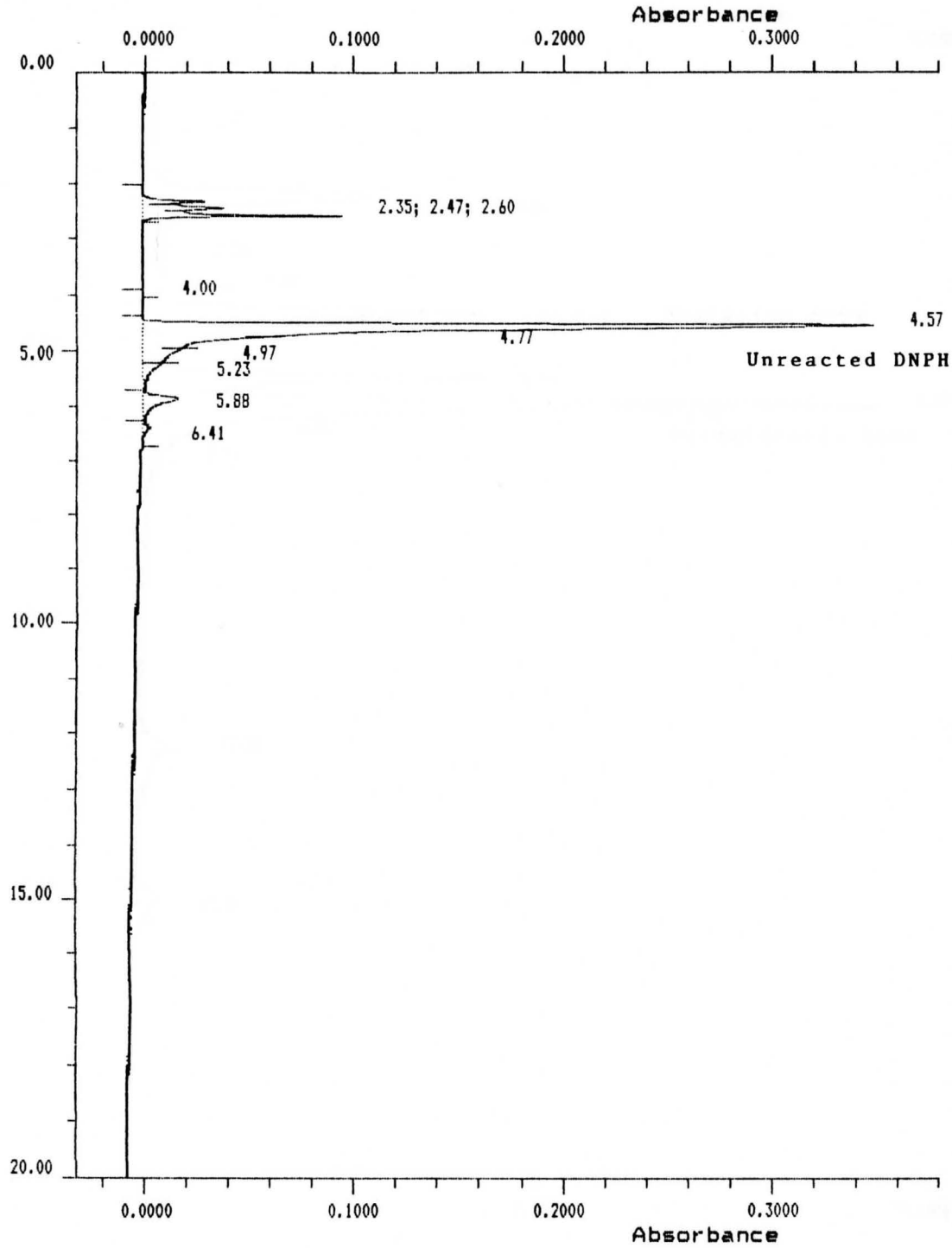


Figure 19: 2,4-DNPH-blank derivative. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 µL injection. UV detection, 345 nm.

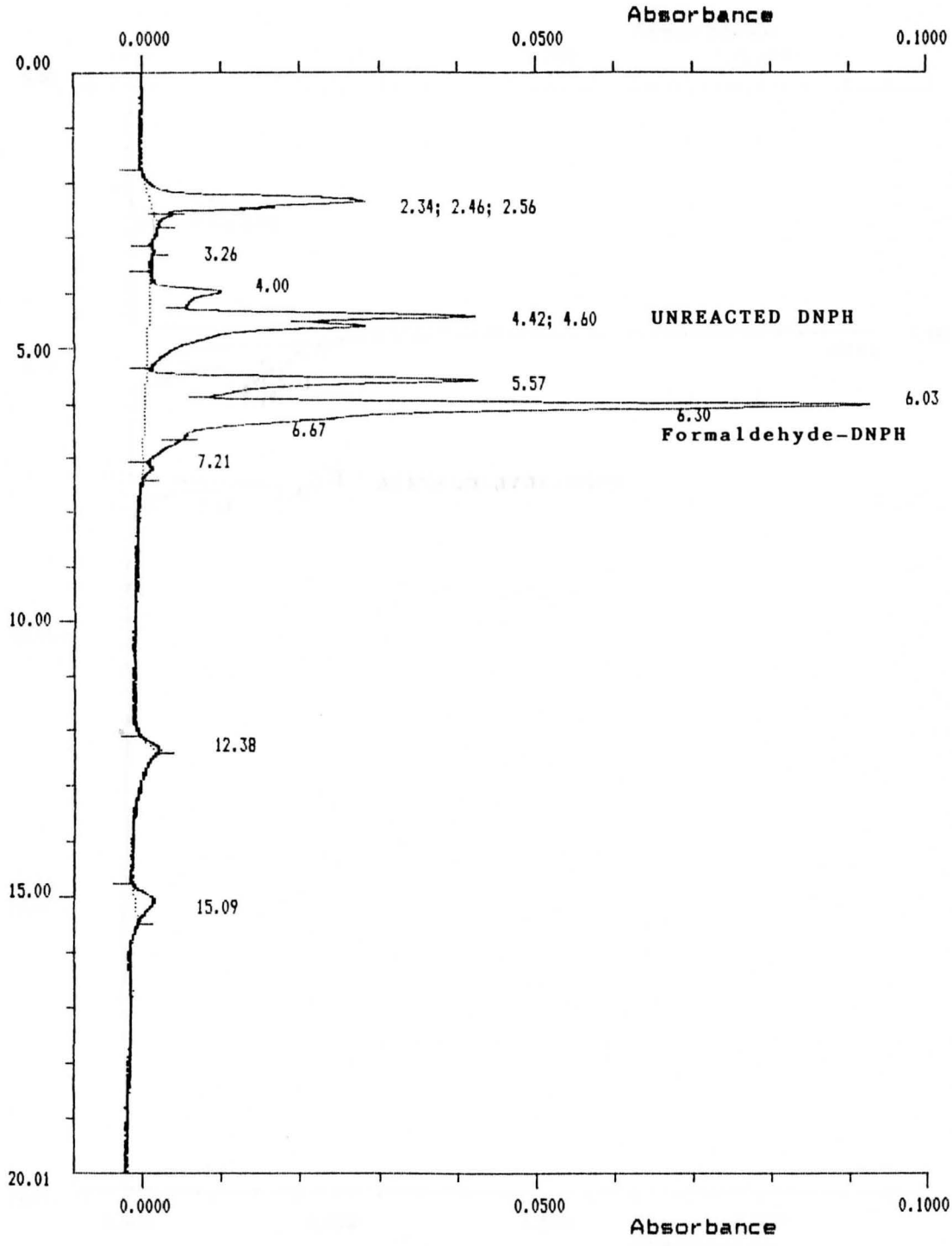


Figure 20: 2,4-DNPH-formaldehyde derivative, concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 µL sample. UV Detection, 345 nm.

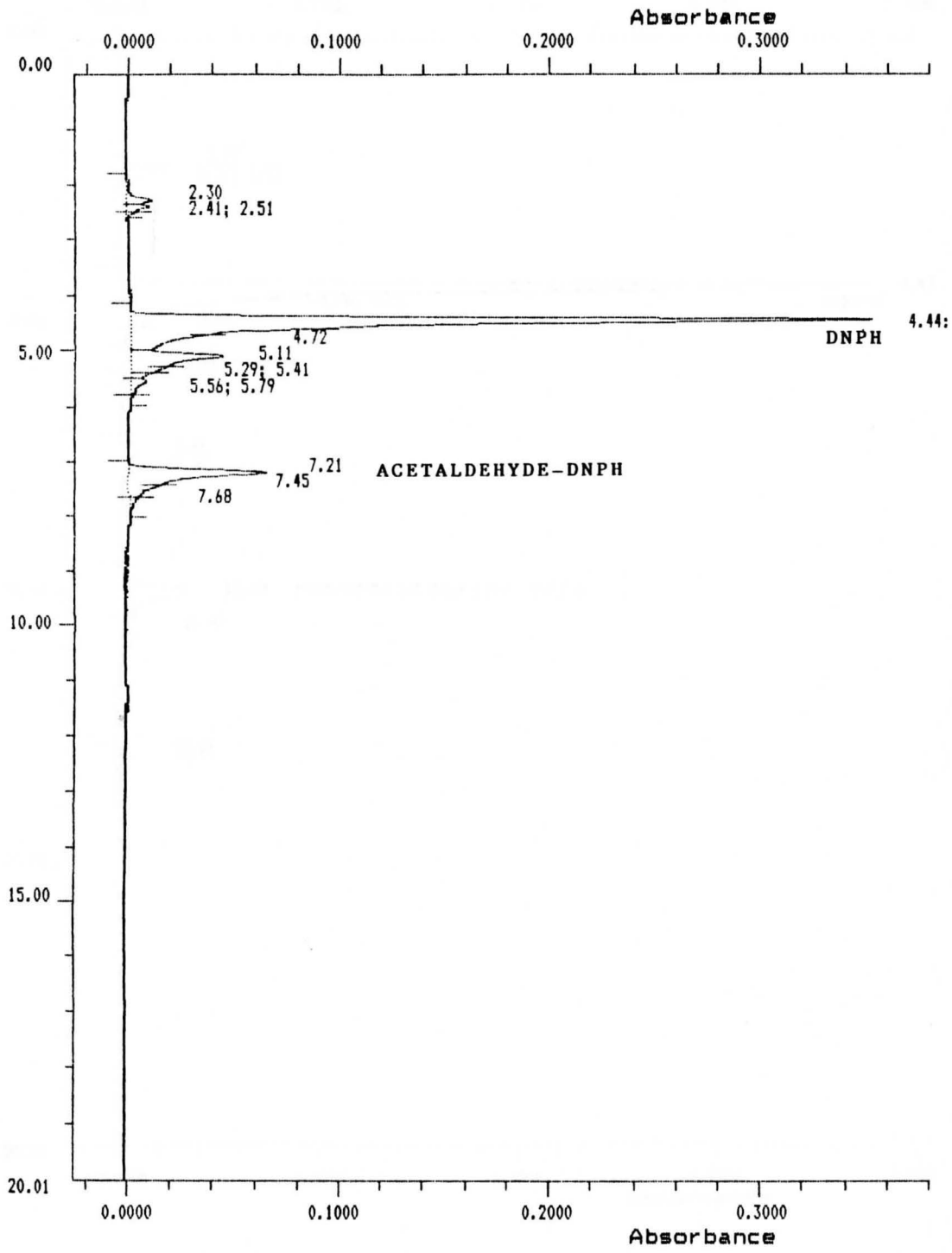


Figure 21: 2,4-DNPH-acetaldehyde derivative, concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 μL sample. UV Detection, 345 nm.

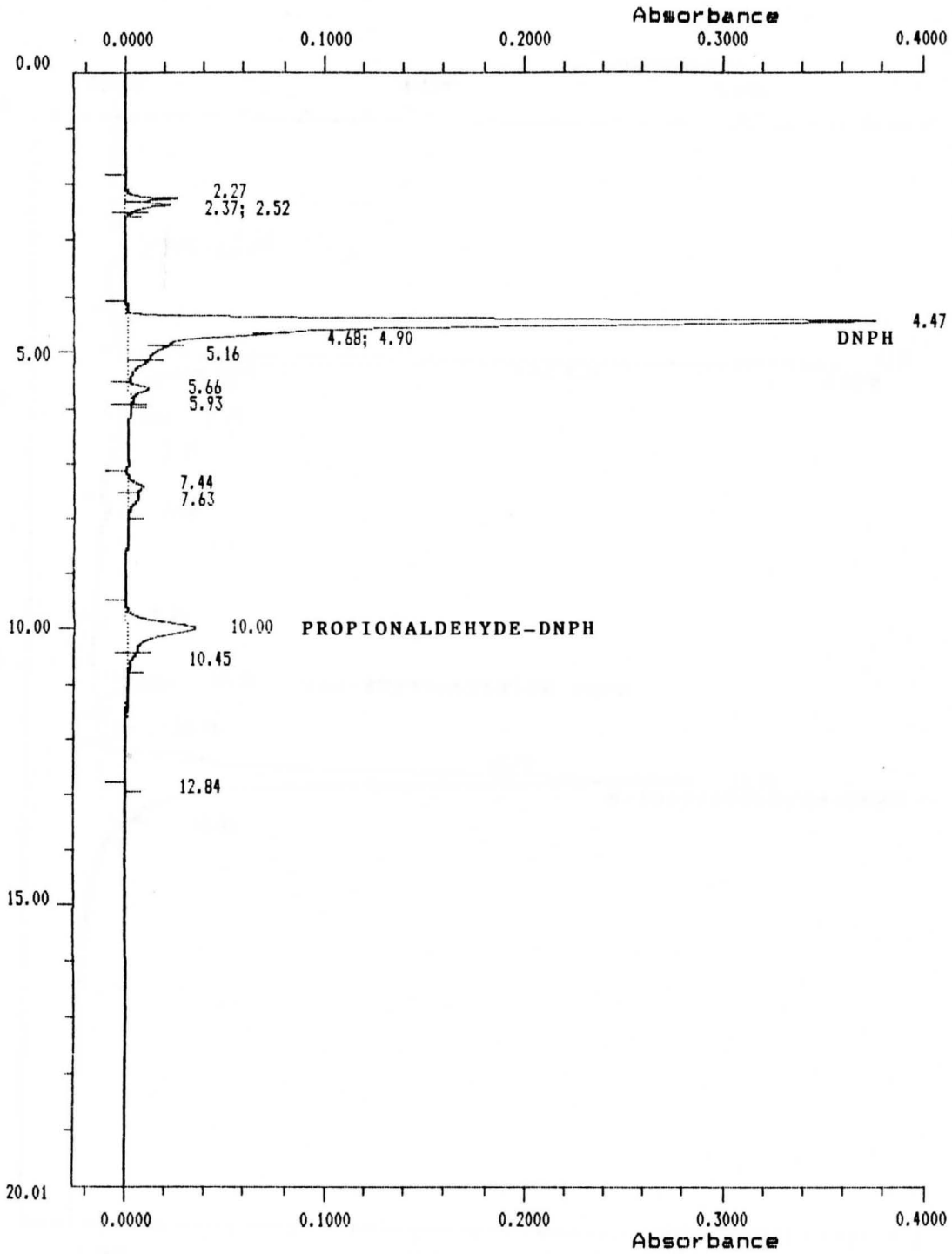


Figure 22: 2,4-DNPH-propionaldehyde derivative, concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 μL sample. UV Detection, 345 nm.

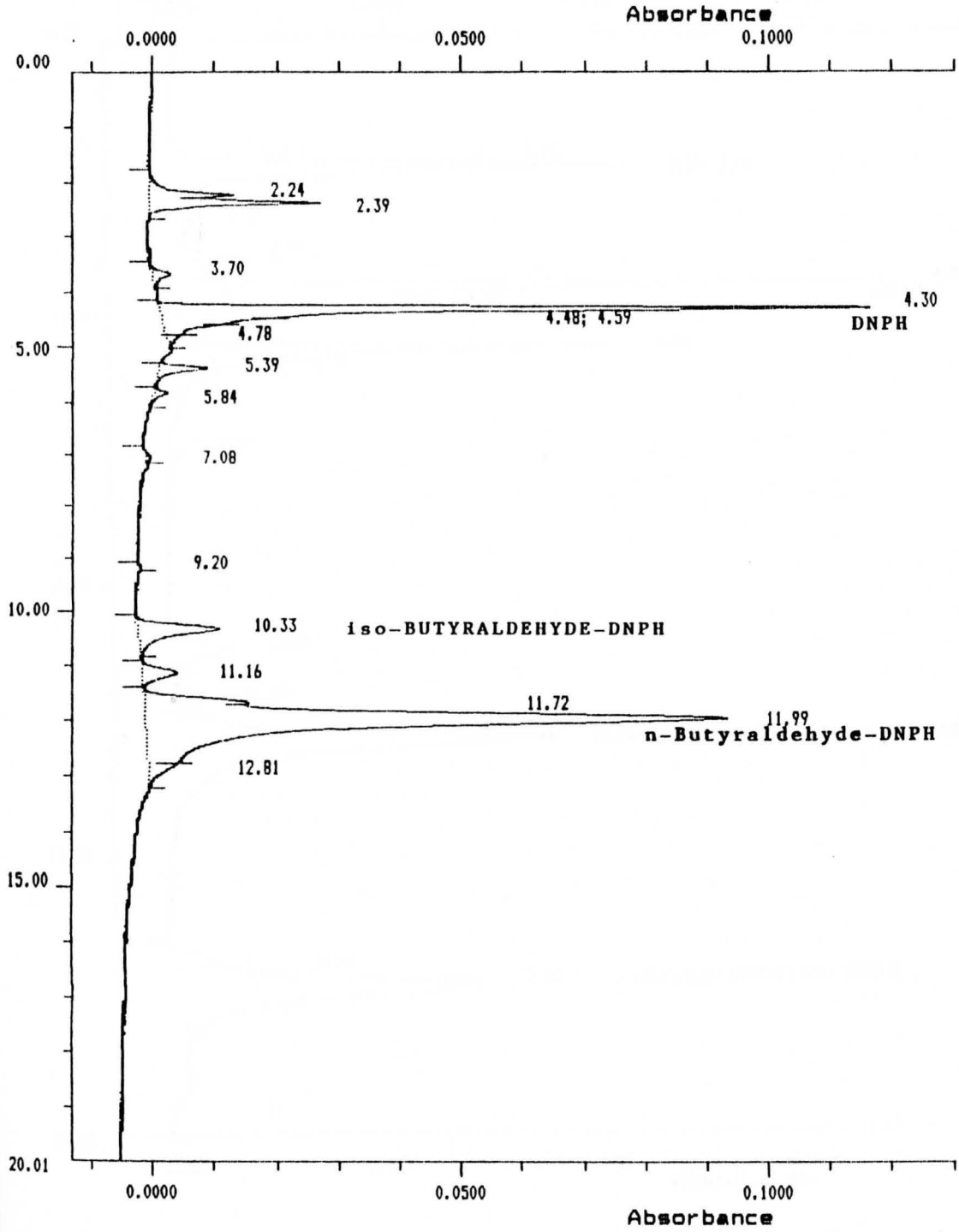


Figure 23: 2,4-DNPH-butyraldehyde derivative, concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 µL sample. UV Detection, 345 nm.

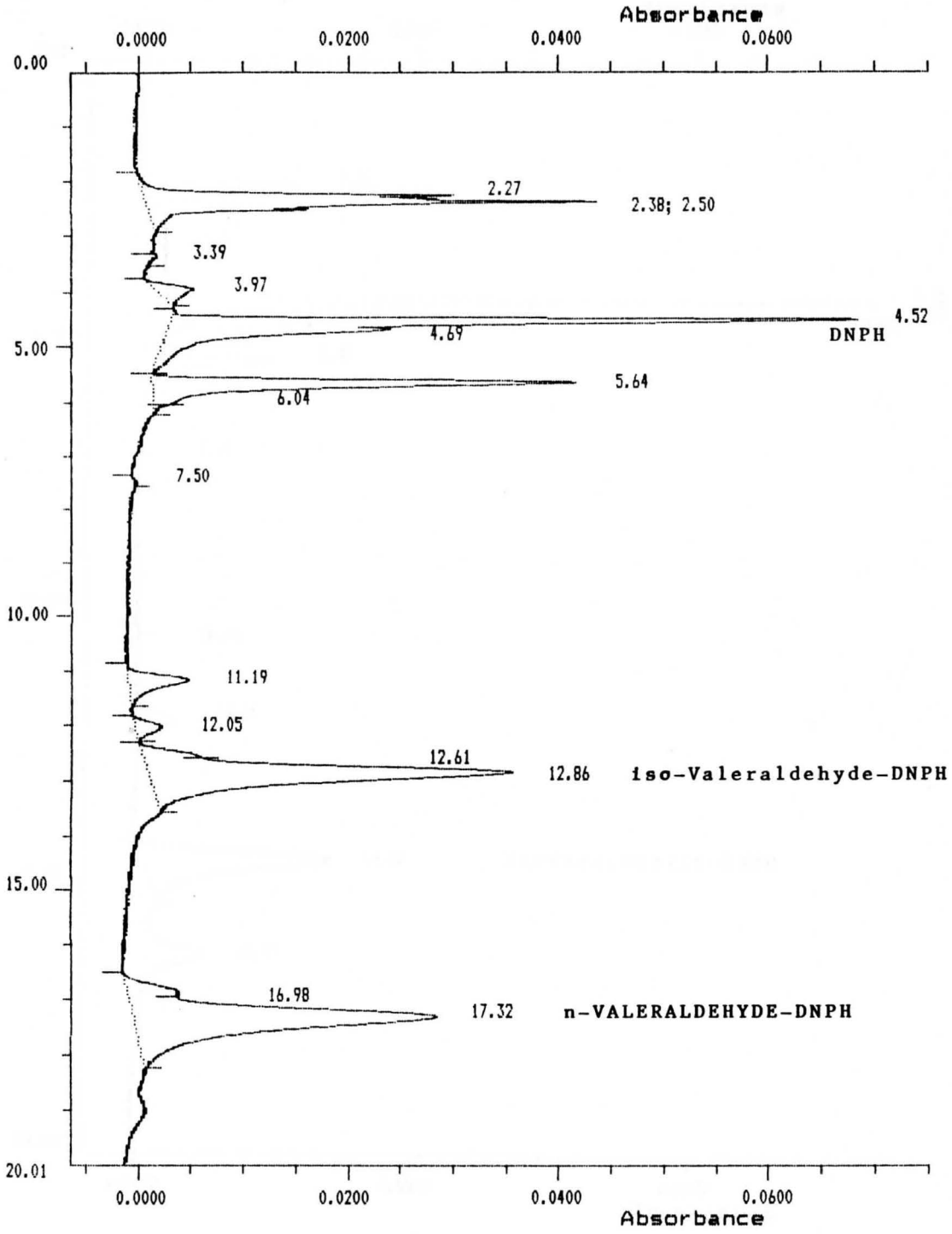


Figure 24: 2,4-DNPH-valeraldehyde derivative, concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 μL sample. UV Detection, 345 nm.

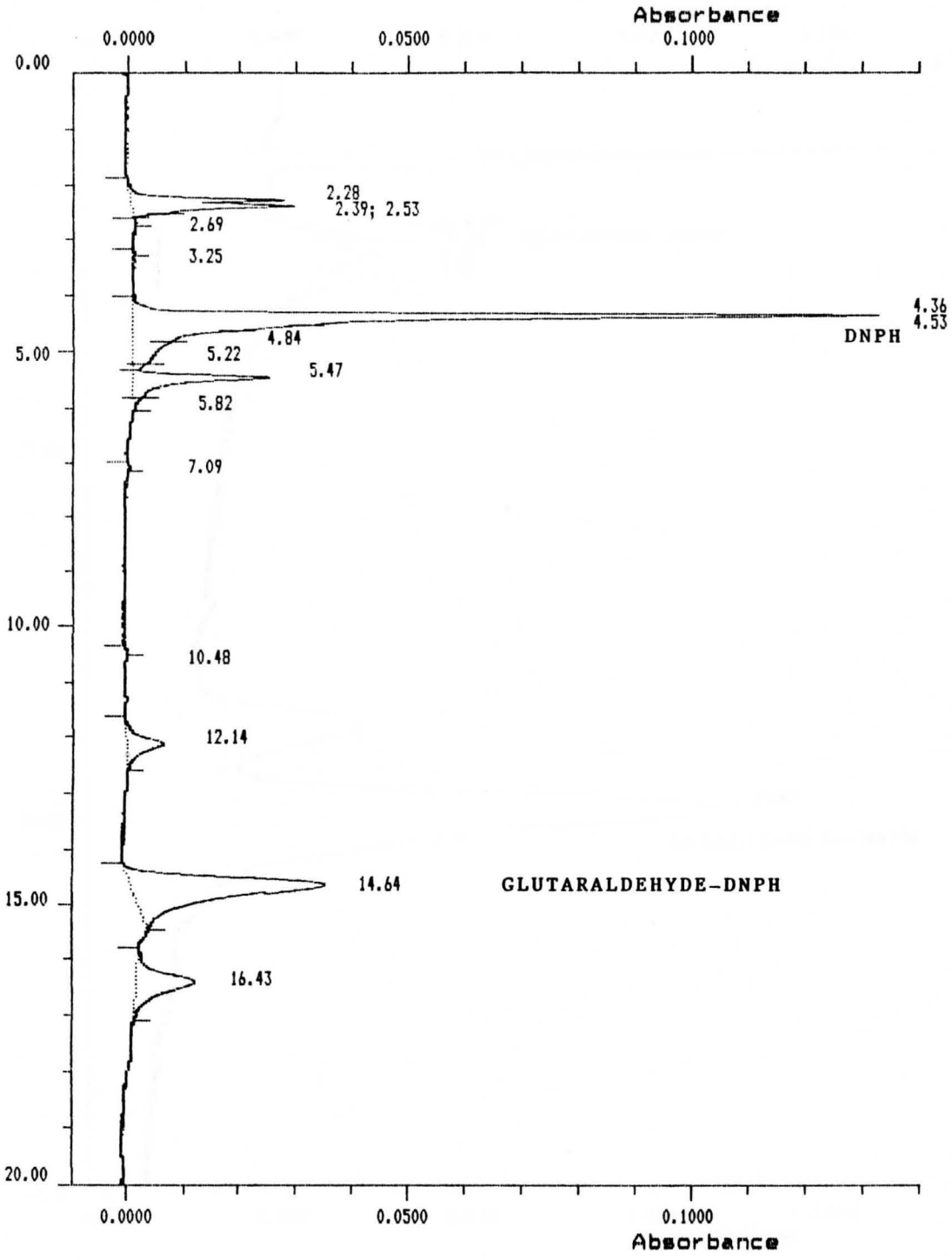


Figure 25: 2,4-DNPH-glutaraldehyde derivative, concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 μL sample. UV Detection, 345 nm.

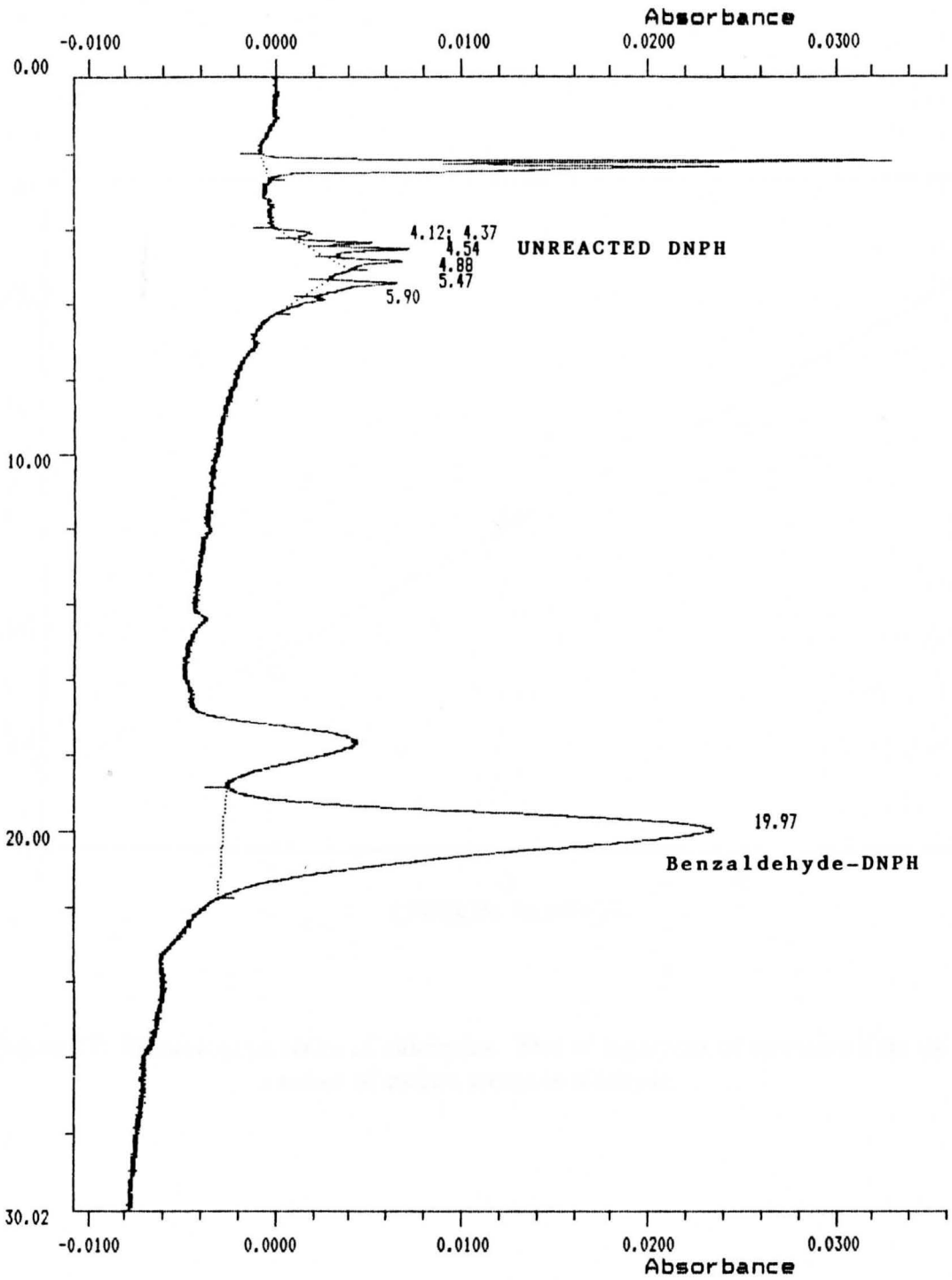


Figure 26: 2,4-DNPH-benzaldehyde derivative, concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 μL sample. UV Detection, 345 nm.

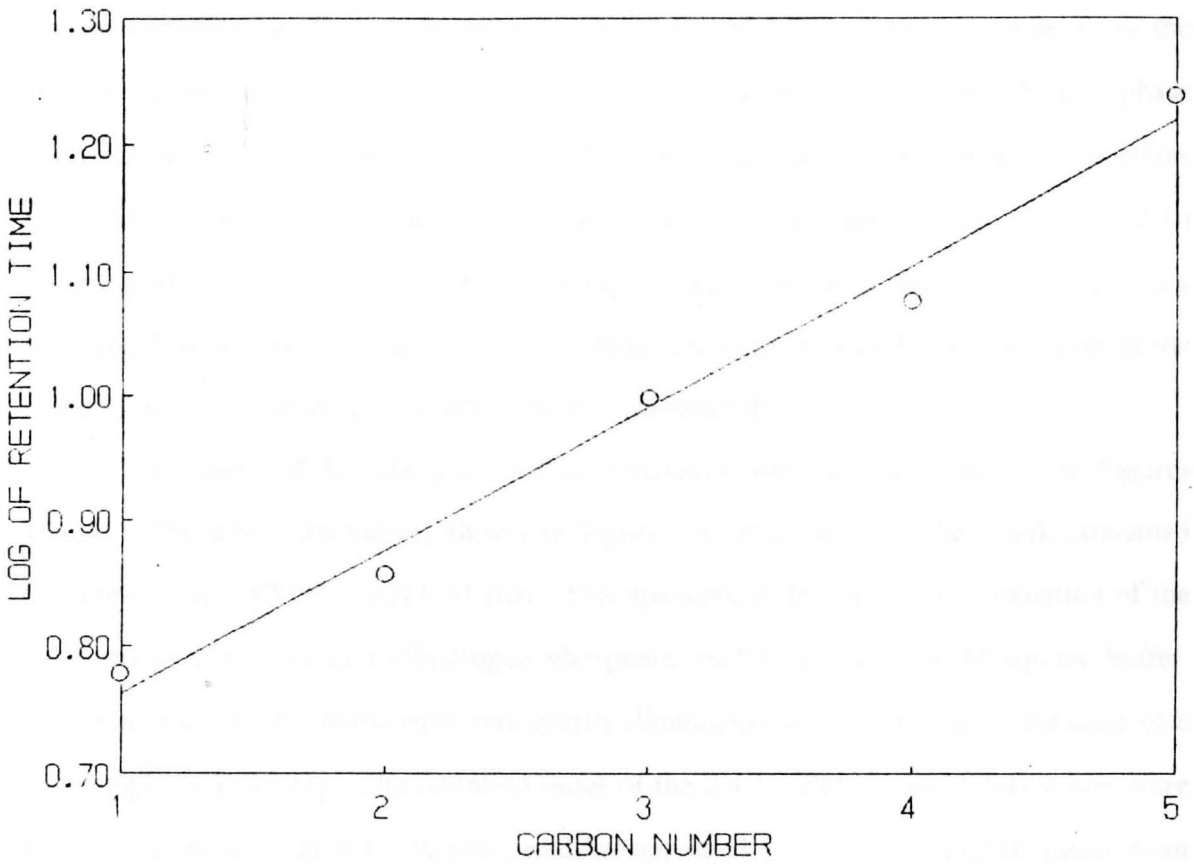


Figure 27: Homologous series of aldehydes. Plot of logarithm of retention time vs. number of carbon atoms in aldehyde.

Separation of 2,4-DNPH-aldehydes HPLC / UV

The mobile phase that gave the best separation of the mixture of aldehydes was determined to be 55% acetonitrile-45% water. This allowed for good resolution of the chromatographic bands without excessive time for all of the bands to elute from the column, as encountered with acetonitrile compositions lower than 55%. Mobile phase compositions consisting of greater than 55% acetonitrile caused the aldehyde derivatives to partition less with the stationary phase, and more with the mobile phase, and to therefore elute from the column before adequate resolution was obtained. The primary wavelength monitored for the 2,4-DNPH-aldehyde derivatives was 345 nm, because of the higher absorbance of the chromophore at that wavelength.

The results of the retention time determination were straightforward (see Figures 19-26). The blank derivative, shown in Figure 19, indicated that the blank contained components at 5.88 min., and 6.41 min. This appeared to be due to contamination of the solutions by the potassium dihydrogen phosphate used to prepare the phosphate buffer. Its occurrence in later blank runs was greatly diminished, corresponding to the used of a new supply of KH_2PO_4. The retention times of the 2,4-DNPH-aldehyde derivatives were easy to recognize because the largest peak that appeared on the chromatogram (aside from the unreacted excess derivative reagent at retention time ~ 4.4 min.) could be attributed to the individual aldehyde derivative that was injected. There appeared to be, however, some sample carry-over. There was evidence of large peaks in the chromatogram of 2,4-DNPH-valeraldehyde (see figure 24). This was attributed to a rearrangement of n-valeraldehyde to iso-valeraldehyde or contamination of the valeraldehyde with iso-valeraldehyde. If rearrangement was occurring, it was not clear whether it was before or after the derivatization. The plot of the homologous series (Figure 27), shows that the retention times observed were in good agreement with the linear theoretical prediction that such a plot produced.

Glutaraldehyde and Benzaldehyde did not derivatize or separate well and were removed from the study. Benzaldehyde formed insoluble clumps in the test tube, which complicated the analysis. It may have simply precipitated in the acetonitrile solution, or its 2,4-DNPH derivative may have rearranged into a more stable structure. Because of the presence of two aldehyde functional groups, glutaraldehyde had the possibility of forming 2 chromatographic peaks. A peak would appear at one retention time if only one aldehyde functional group was derivatized. If both aldehyde groups were derivatized, the peak would appear at a larger retention time. This was due to the less polar characteristics of the doubly derivatized glutaraldehyde, resulting in stronger interaction with the reversed phase column. There also was a possibility of many side reactions occurring, creating a complicated mixture of related organic products. This aldehyde was thus removed from the study.

Post column zinc reduction

UV Spectra in Bulk Solution

UV Spectra were obtained for 2,4-DNPH derivatives of formaldehyde, acetaldehyde and propionaldehyde, and were compared with 2,4-DAPH aldehyde derivatives that were reduced in bulk solution using zinc dust. The spectra of the aldehyde derivatives demonstrated different absorption maxima. The bulk solution spectra will not be shown for either compound, however, since these spectra are shown for diode array scans taken during chromatography and are essentially identical.

UV Spectra from the Beckman Diode Array

The chromatogram for the unreduced 2,4-DNPH-aldehyde mixture is shown in Figures 28, with UV detection at 345 nm. The chromatogram obtained from the separation of the same derivative mixture, only using a zinc reduction column with UV detection at 345 nm is shown in Figures 29. Diode array scans of UV spectra at retention

times of the 2,4-DNPH-acetaldehyde derivative (7.13 - 7.16 min. for the unreduced form, and 7.17 - 7.20 min. for the reduced form) are shown in Figures 30. They were superimposed for comparison purposes.

When the zinc-packed bed reactor was connected to the chromatography system, the dead volume increased slightly, but the retention times of the aldehyde derivatives decreased. This was not due to the zinc bed reactor, however, but was due to the installation of a new analytical column at that point of the project. The retention time differences between the columns were slight, but became significant when relatively longer retention times were involved, such as with the valeraldehyde derivative.

The bed reactor was of the zero-retention volume type, but there was still evidence of band broadening due to the reactor, mainly attributed to the length and the diameter of the reactor tube (see theory section). This can be seen in the increase in the system dead volume (retention peak 2.43 to 2.49 min). Since this was post-column derivatization, any relative change in the retention times must be attributed to other factors than the on-line reduction, because the separation occurred before the reduction.

The UV spectra shown in Figure 30 were of the most interest, with all 2,4-DNPH derivatives showing similar behavior. The solid line on the Figure shows the spectrum of 2,4-DNPH acetaldehyde, while the dashed line shows the absorbance spectrum of the reduced 2,4-DNPH-acetaldehyde (i.e., 2,4-DAPH acetaldehyde). It is evident from a comparison of these spectra that the production of the reduced form should present itself on a chromatogram with a decreased absorbance at 345 nm and an increased absorbance at 212 nm. Some data were taken at 254 nm, but the difference in absorbance between the reduced form and the unreduced form appears to be negligible at this wavelength. Data were taken at 254 nm for comparison to literature separations. The difference in absorbance at 212 nm vs. 345 nm was also of great utility for peak identification since only 2,4-DNPH aldehyde derivatives would show this behavior.

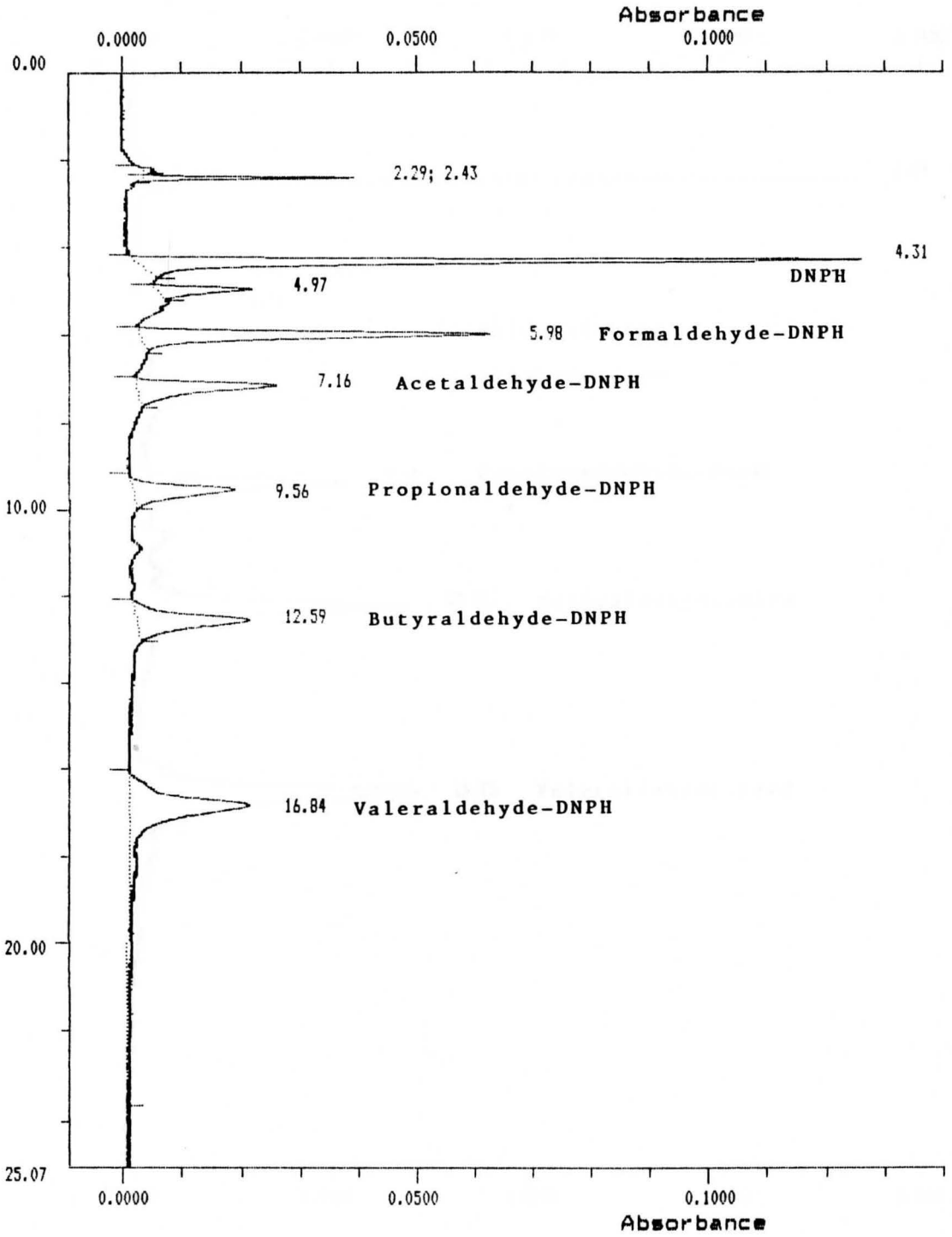


Figure 28: 2,4-DNPH-aldehyde mixture derivatives (formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, valeraldehyde), concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 μL sample. UV Detection, 345 nm.

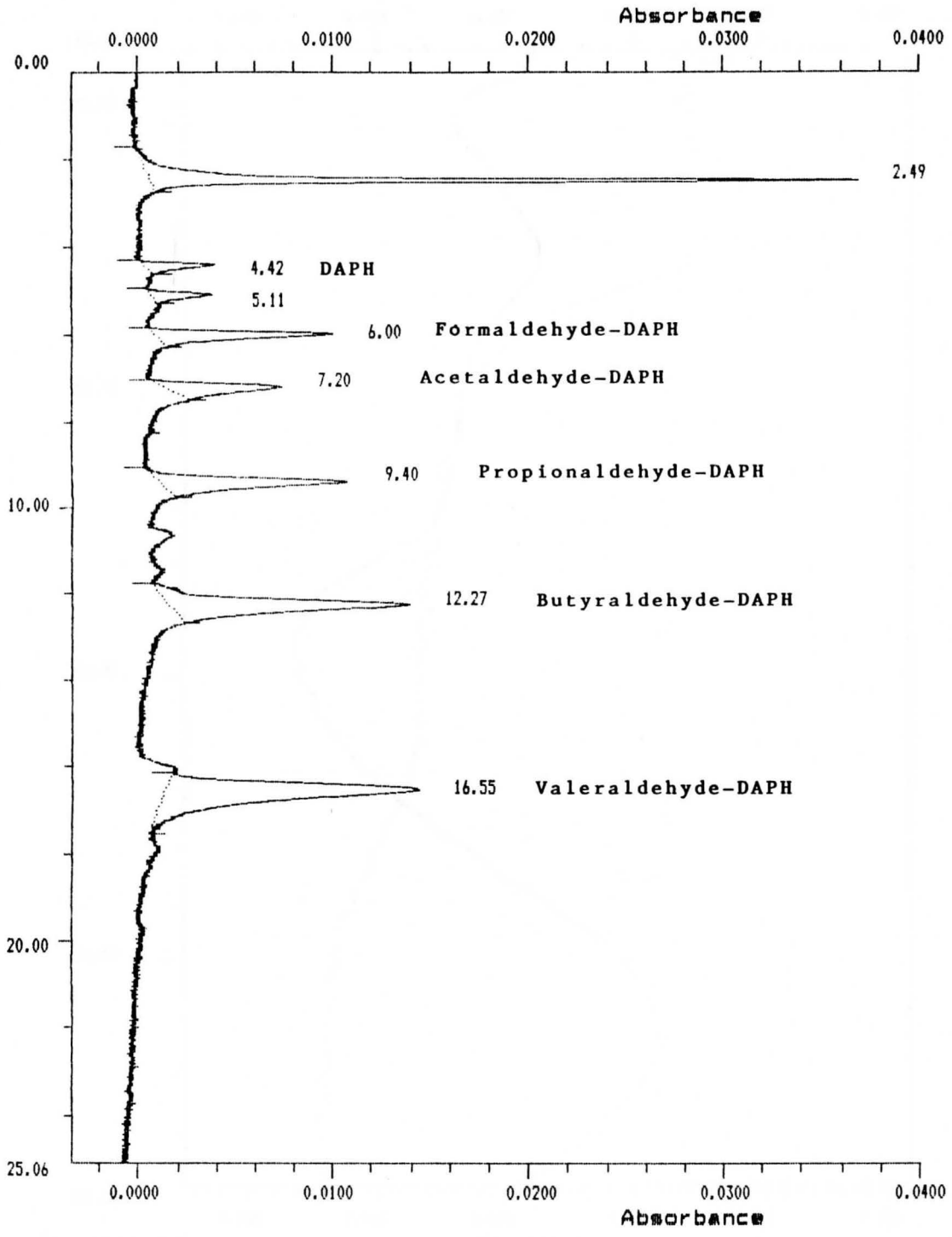


Figure 29: 2,4-DNPH-aldehyde mixture derivatives (formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, and valeraldehyde), concentration: 6.17×10^{-4} M. Mobile Phase: 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Ambient Temperature. 10 µL sample. On-line reduction -- zinc bed reactor. UV Detection, 345 nm.

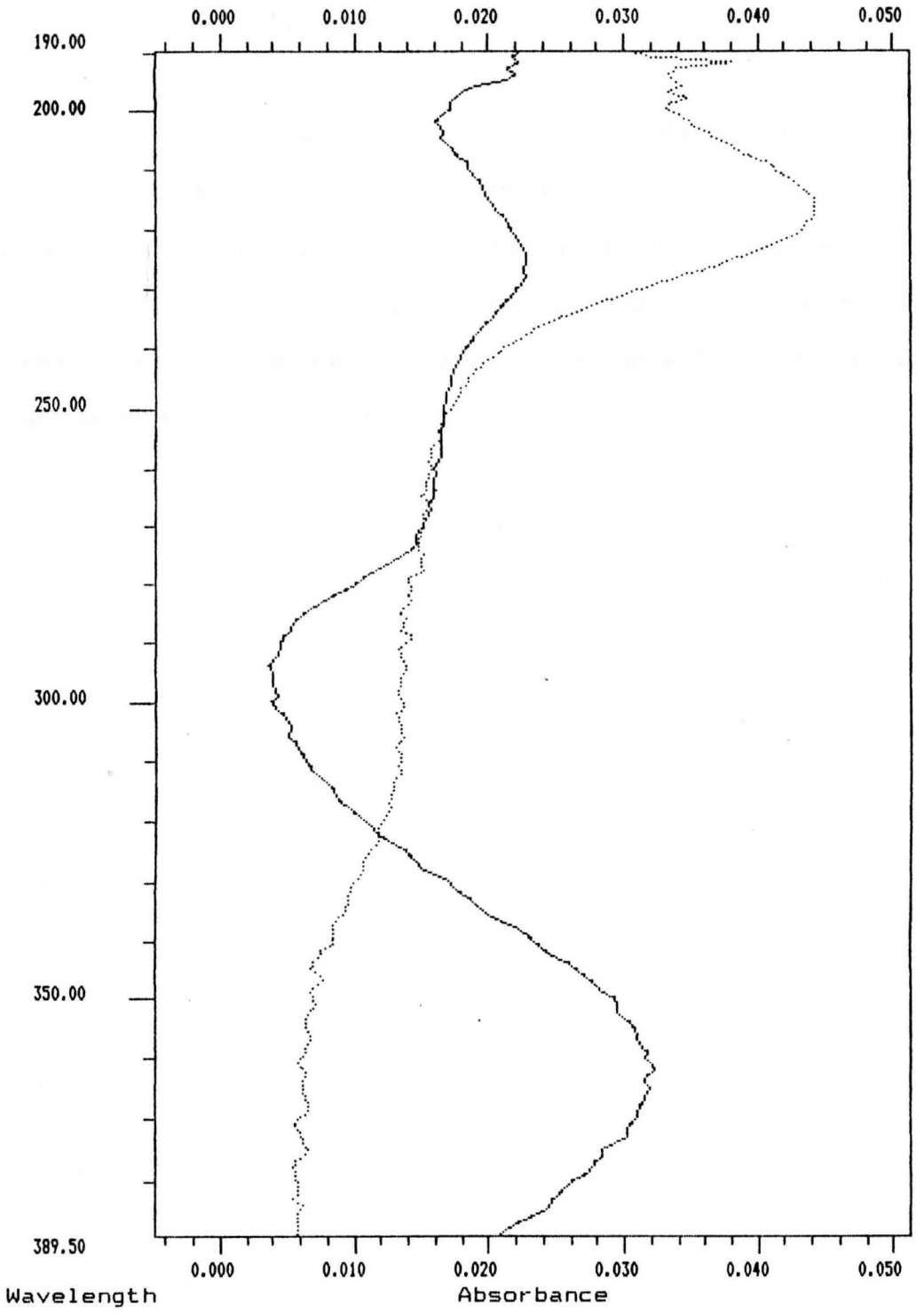


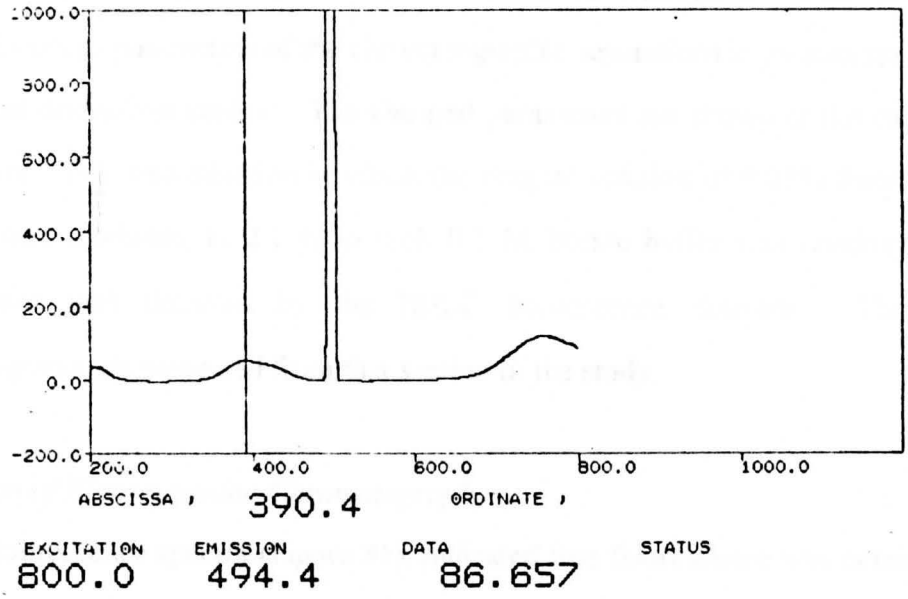
Figure 30: UV Spectra of 2,4-DNPH- and 2,4-DAPH-acetaldehyde derivatives. The diode array scans taken by the Beckman UV detector at retention times 7.13-7.20 min. were superimposed for comparison. The solid line corresponds to DNPH derivatives, the dashed line represents DAPH derivatives reduced on-line with a zinc bed reactor.

Results of Fluorescamine Derivative

Fluorescence spectra

The excitation and emission spectra of the reduced-in-bulk and fluorescence-derivatized 2,4-DNPH-acetaldehyde are shown in Figure 31. The spectra show the wavelength of maximum excitation and the wavelength of maximum emission, which were 390 nm and 494 nm, respectively. The fluorescence spectra of the formaldehyde and propionaldehyde derivatives were similar to those obtained for the acetaldehyde derivative, and therefore are not shown.

(a)



(b)

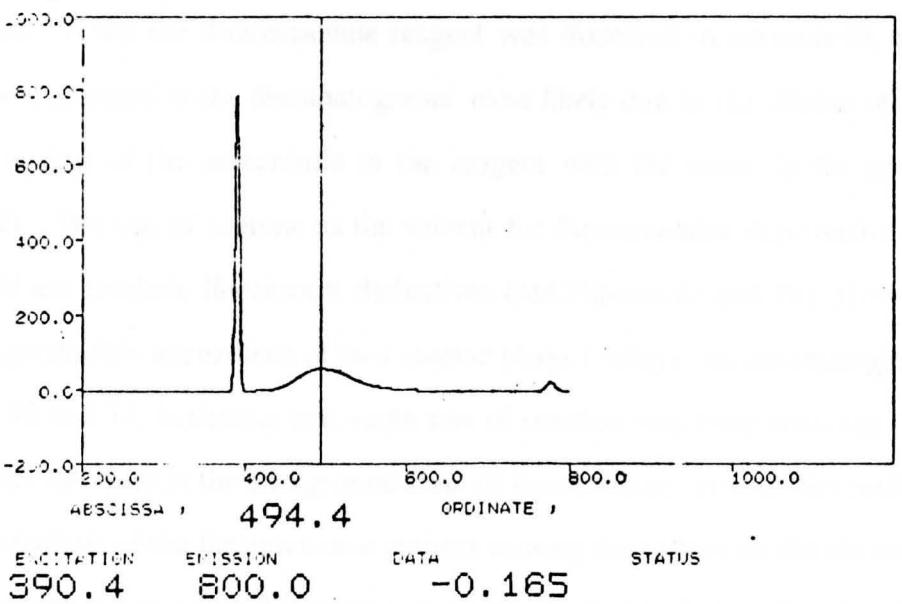


Figure 31: (a) Excitation (390 nm max) and (b) Emission (494 nm max) Spectra of acetaldehyde-2,4-DAPH-fluorescamine derivative. Acetaldehyde concentration: 6.17 x 10^{-4} M. Derivatized in bulk solution with excess fluorescamine.

HPLC Reaction Conditions

The chromatograms presented in Figures 32 through 34 were the result of changing various parameters of the chromatographic separations in an attempt to produce fluorescent derivatives on-line. The changed parameters are shown in the caption under each figure. The determination in which the reagent solution of 0.05% fluorescamine in acetone was combined in 2:1 ratio with 0.1 M borate buffer was conducted, but no fluorescence was detected by the HPLC fluorescence detector. Therefore, no chromatograms are presented from that section of the study.

Discussion of Fluorescamine Chromatography

Fluorescence spectra (Figure 31), indicated that fluorescence was occurring in the derivative solutions. However, in those studies, the fluorescamine reagent was added in excess and at conditions that were not necessarily optimum for reaction. When it was attempted to transfer this process to perform on-line derivatization, fluorescence could not be detected. When the fluorescamine reagent was dissolved in acetonitrile, an unstable baseline was obtained in the chromatograms, most likely due to the release of air bubbles from the mixing of the acetonitrile in the reagent with the water in the mobile phase (Figure 32). The use of acetone as the solvent for fluorescamine improved the baseline, but still did not produce fluorescent derivatives (see Figures 33 and 34). However, there was the reproducible appearance of two reverse peaks (valleys) on chromatograms shown in Figures 32 and 34, indicating that some sort of reaction may have occurred that caused a momentary decrease in the background level of fluorescence. It was very unlikely that it was the hydrolysis of the fluorescamine reagent causing the valleys on the chromatograms, since fluorescamine in a non-fluorescing reagent, and it is hydrolyzed to non-fluorescing products.

The prospect of buffering the mobile phase with borate buffer to pH 9, followed by a mixing coil reactor seemed promising, but also did not produce a fluorescence response.

It may have been that the reagent was being hydrolyzed in the presence of the water in the borate buffer before it even had a chance to react with any type of primary amino group. Another reason that the reaction did not produce fluorescence may have been due to the quenching effect of the nitro groups. If one of the nitro groups on the phenylhydrazone structure was not reduced to an amino group, its presence could have resulted in n —> p* electronic transitions. This would have resulted in intersystem crossing and diminished fluorescence.

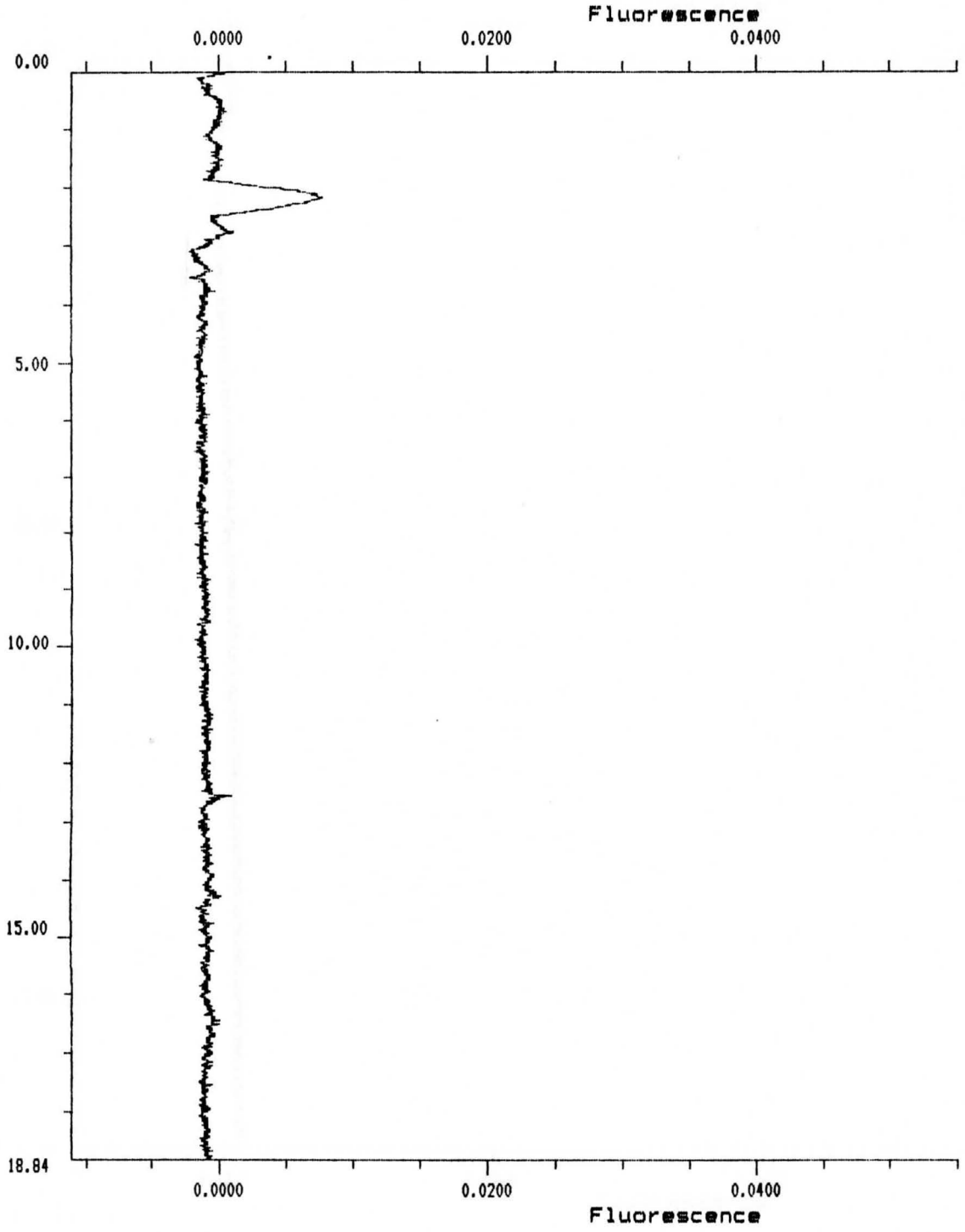


Figure 32: Small zinc particle size with 50% acetonitrile-50% water mobile phase. Sample: 2,4-DNPH-aldehyde mixture, concentration 6.17 x 10^{-6} M. Mobile phase: 50% acetonitrile-50% water. Flow rate: 1.00 mL/min. Ambient temperature. Sample size: 10 μL. Zinc particle: 325 mesh. Reagent: 0.050% Fluorescamine in 50% acetonitrile-50% water. Reagent Flow rate: 1.00 mL/ min. Fluorescence detection.

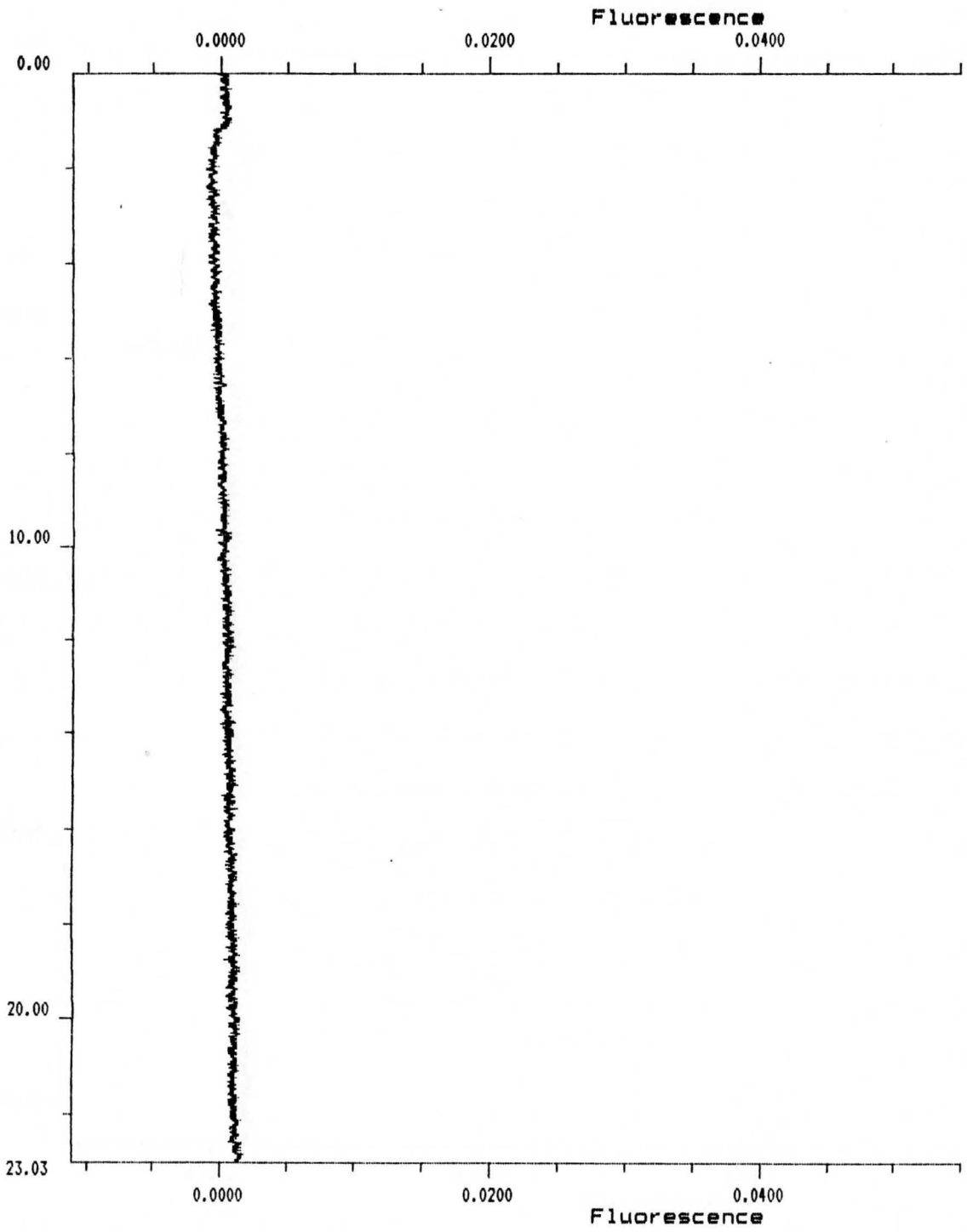


Figure 33: Higher concentration of aldehyde with 325 mesh zinc particle, reagent in acetone. Sample: 2,4-DNPH-aldehyde mixture, concentration 6.17 x 10^{-4} M. Mobile phase: 50% acetonitrile-50% water. Flow rate: 1.00 mL/min. Ambient temperature. Sample size: 10 µL. Zinc particle: 325 mesh. Reagent: 0.050% Fluorescamine in acetone. Reagent Flow rate: 1.00 mL/ min. Fluorescence detection.

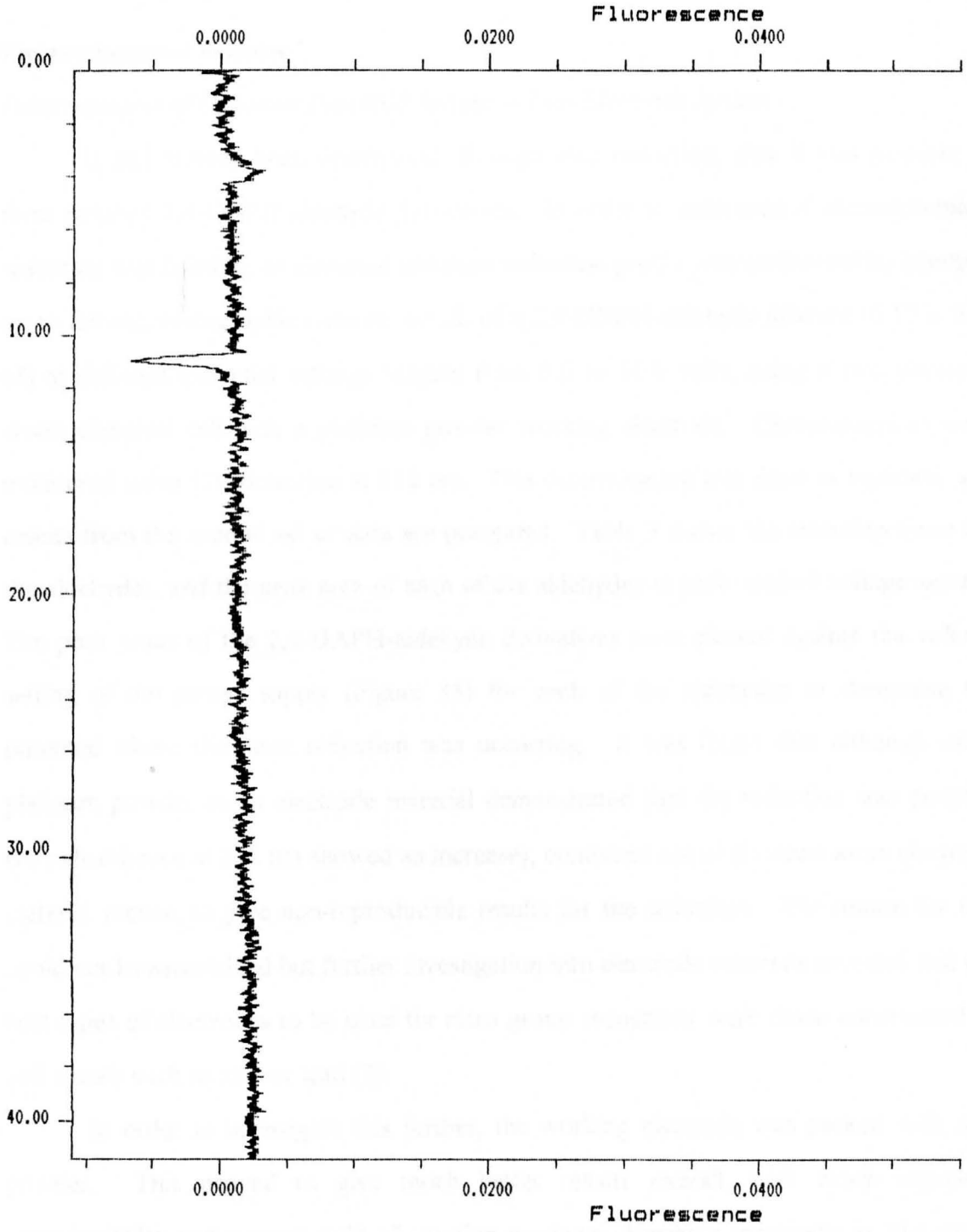


Figure 34: More polar mobile phase, 2 foot reaction coil. slow flow rate, Reagent dissolved in acetone. Sample: 2,4-DNPH-aldehyde mixture, concentration 6.17 x 10^{-4} M. Mobile phase: 65% acetonitrile-35% water. Flow rate: 0.70 mL/min. Ambient temperature. Sample size: 10 µL. Zinc particle: 325 mesh. Reagent: 0.050% Fluorescamine in acetone. Reagent Flow rate: 0.70 mL/ min. 2.0 foot reaction coil. Fluorescence detection.

Electrochemical Reactor

Determination of Optimum Potential Setting -- Two-Electrode System

It had already been determined, through zinc reduction, that it was possible to form reduced 2,4-DNPH-aldehyde derivatives. In order to determine if electrochemical reduction was feasible, an electrical potential reduction profile was performed by injecting on to the chromatographic column, 10 μL of a 2,4-DNPH-aldehyde mixture (6.17×10^{-4} M) at different potential settings ranging from 0.0 to 50.0 volts, using a two electrode electrochemical cell with a platinum powder working electrode. Chromatograms were monitored using UV detection at 212 nm. This determination was done in replicate, and results from the second set of data are presented. Table 3 shows the retention times for the aldehydes, and the peak area of each of the aldehydes at each applied voltage setting. The peak areas of the 2,4-DAPH-aldehyde derivatives were plotted against the voltage setting of the power supply (Figure 35) for each of the aldehydes to determine the potential where the most reduction was occurring. It was found that although using platinum powder as an electrode material demonstrated that the reduction was possible (i.e., absorbance at 212 nm showed an increase), continued use of platinum as an electrode material proved to give non-reproducible results for the reduction. The reason for this could not be ascertained but further investigation into electrode materials revealed that the best types of electrodes to be used for nitro group reductions were those constructed of soft metals such as zinc or lead (5).

In order to investigate this further, the working electrode was packed with zinc powder. This proved to give much better results overall, with much improved reproducibility and a good yield of reaction products absorbing maximally at 212 nm at various applied potentials. Data were taken at applied potentials ranging from 0 to 70 volts and the data are presented in Table 4. From this data it is clear that at 30 volts and at 50 volts reduction is occurring to give products that absorb at the desirable wavelength of 212 nm. The chromatogram obtained for the 2,4-DNPH aldehyde mixture at the 50.0

volt potential setting is shown in Figure 36. The solid line on the chromatogram represents UV detection at 345 nm and the dashed line detection at 212 nm. Figure 37 shows, for comparison purposes, the same sample with no applied potential (the electrochemical cell was removed).

To further clarify the differences between the derivatives, the data were graphed in Figure 38 as applied potential versus absorbance at 212 nm and 345 nm for 2,4-DNPH acetaldehyde and in Figure 39 as applied potential versus the ratio of the absorbances at the two wavelengths (212 nm/345 nm). From these graphs it was very clear that the reduction was occurring as predicted and it was very evident that the applied potential played a key role in determination of the product or mix of products obtained from the electrochemical reduction. Again, it is quite evident that 30 volts and 50 volts are the potentials of interest. the electrochemical cell removed.

The data obtained using the platinum electrode material appeared to mirror that produced using the zinc electrode. There were two maxima evident (15 volts and 30 volts) with a minimum in between. It appeared likely that a similar mechanism was occurring and that the difference in applied potential was due to the choice of electrode material. Interpretation of both sets of data is thus similar. The first maximum can be explained by the transformation of one of the 2,4-DNPH-aldehyde derivative nitro groups to an amino group. The minimum represents the oxidation (or partial oxidation) of this amino group back to a nitro group at the auxiliary electrode. The second maximum represents the oxidation of both nitro groups to amino groups and the second minimum (i.e., at 50 volts for platinum and at 70 volts for zinc) is the reoxidation of both groups by the auxiliary electrode.

In Figure 36, therefore, using zinc as the electrode material at a 50 volt applied potential, it is clear that there was a significantly higher absorbance at 212 nm than at 345 nm. This means that a greater portion of the DNPH sample was converted to DAPH at

the working electrode. The greater absorbance at 212 nm agrees with those results obtained using a zinc bed reactor.

One other noteworthy observation from this data was that if the absorbances at the wavelength maxima for the 2,4-DNPH-aldehyde and the 2,4-DAPH-aldehyde are compared, there is little difference between them. Thus based upon this fact, it would appear that the detection limits for the reduced derivatives should be similar to those for the non-reduced derivatives and quantitative analysis accuracy, precision, and sensitivity should not suffer very little, if any, from electrochemically reducing the derivatives.

Table 3: Peak Areas and Voltage settings of Chromatograms 2,4-DNPH-aldehyde derivatives obtained using platinum as the electrode material. The wavelength of detection was 212 nm.

Data for Aldehyde Peak Area Vs. Voltage setting.

	Aldehyde Derivative Peak Area				
Voltage	Form	Acet	Prop	Butyr	Valer
0.0	7.293	5.290	3.671	4.841	6.374
10.0	7.851	5.617	4.671	8.627	13.294
20.0	6.943	5.241	3.794	7.251	10.104
25.0	7.055	5.833	4.520	7.463	11.949
30.0	9.143	6.903	6.027	10.530	15.207
40.0	8.990	6.662	5.122	9.058	14.359
50.0	7.994	5.793	5.062	9.093	13.120

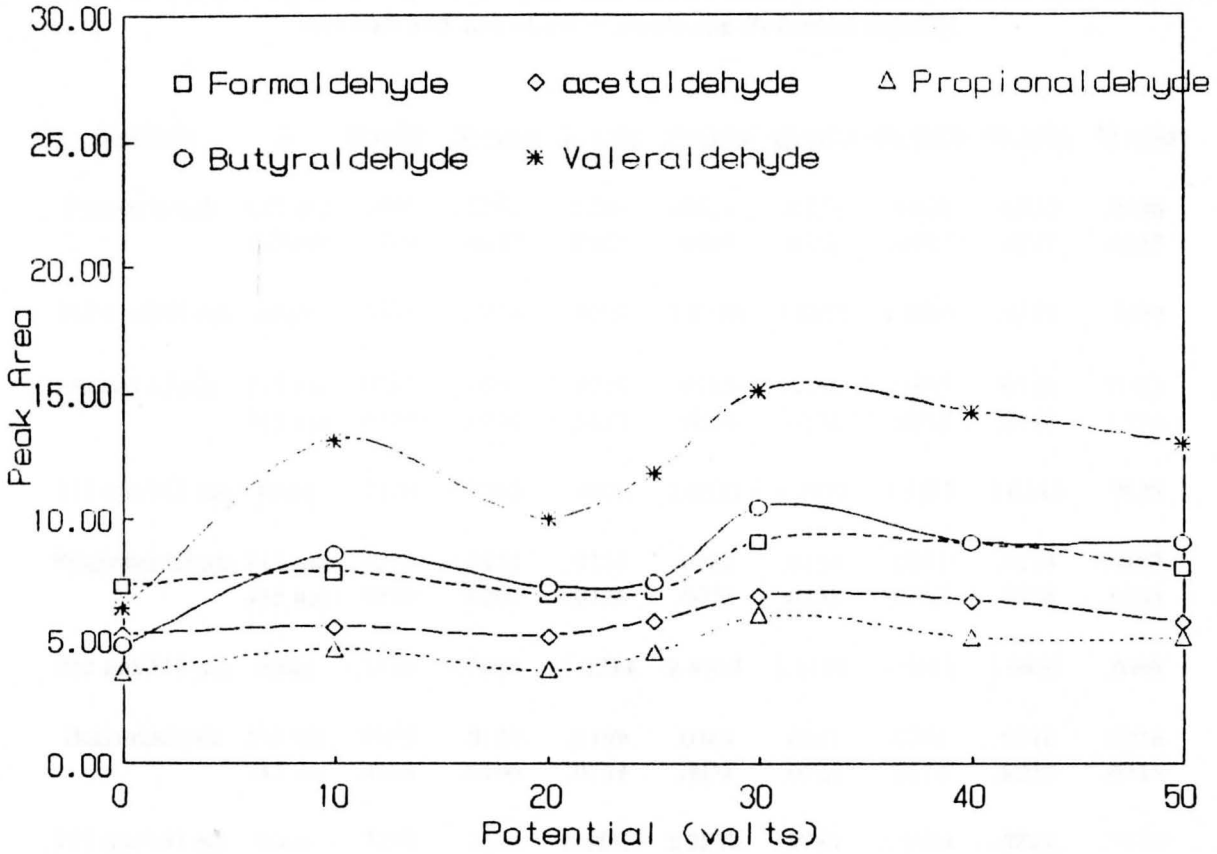


Figure 35: Plot of peak area of aldehyde derivatives at 212 nm vs. power supply voltage using platinum as the electrode material.

Table 4: Peak Heights of Aldehyde Derivatives and Ratios of Aldehyde Derivatives at 212 nm and 345 nm of Zinc Electrode Potential Profile.

Aldehyde	λ	0 volts	10 volts	20 volts	30 volts	40 volts	50 volts	60 volts	70 volts
Formaldehyde	212 nm	.0606	.0368	.0241	.0439	.0358	.0434	.0230	.0436
	345 nm	.0929	.0497	.0267	.0193	.0196	.0087	.0241	.0615
212 nm/345 nm	Ratio	.6523	.7404	.9026	2.2746	1.8265	4.9885	.9544	.7089
Acetaldehyde	212 nm	.0237	.0162	.0109	.0183	.0165	.0206	.0116	.0185
	345 nm	.0326	.0196	.0121	.0090	.0135	.0055	.0110	.0236
212 nm/345 nm	Ratio	.7270	.8265	.9008	2.0333	1.2222	3.7455	1.0545	.7839
Propionaldehyde	212 nm	.0219	.0154	.0135	.0182	.0154	.0241	.0134	.0188
	345 nm	.0279	.0202	.0126	.0073	.0138	.0068	.0128	.0233
212 nm/345 nm	Ratio	.7849	.7624	1.0714	2.4932	1.1159	3.5441	1.0469	.8069
Butyraldehyde	212 nm	.0195	.0155	.0199	.0169	.0181	.0384	.0210	.0226
	345 nm	.0269	.0206	.0134	.0074	.0185	.0114	.0237	.0316
212 nm/345 nm	Ratio	.7249	.7524	1.4851	2.2838	.9784	3.3684	.8861	.7152
Valeraldehyde	212 nm	.0193	.0186	.0281	.0196	.0186	.0501	.0298	.0296
	345 nm	.0255	.0190	.0265	.0083	.0244	.0141	.0319	.0354
212 nm/345 nm	Ratio	.7569	.9789	1.0604	2.3614	.7623	3.5532	.9342	.8362

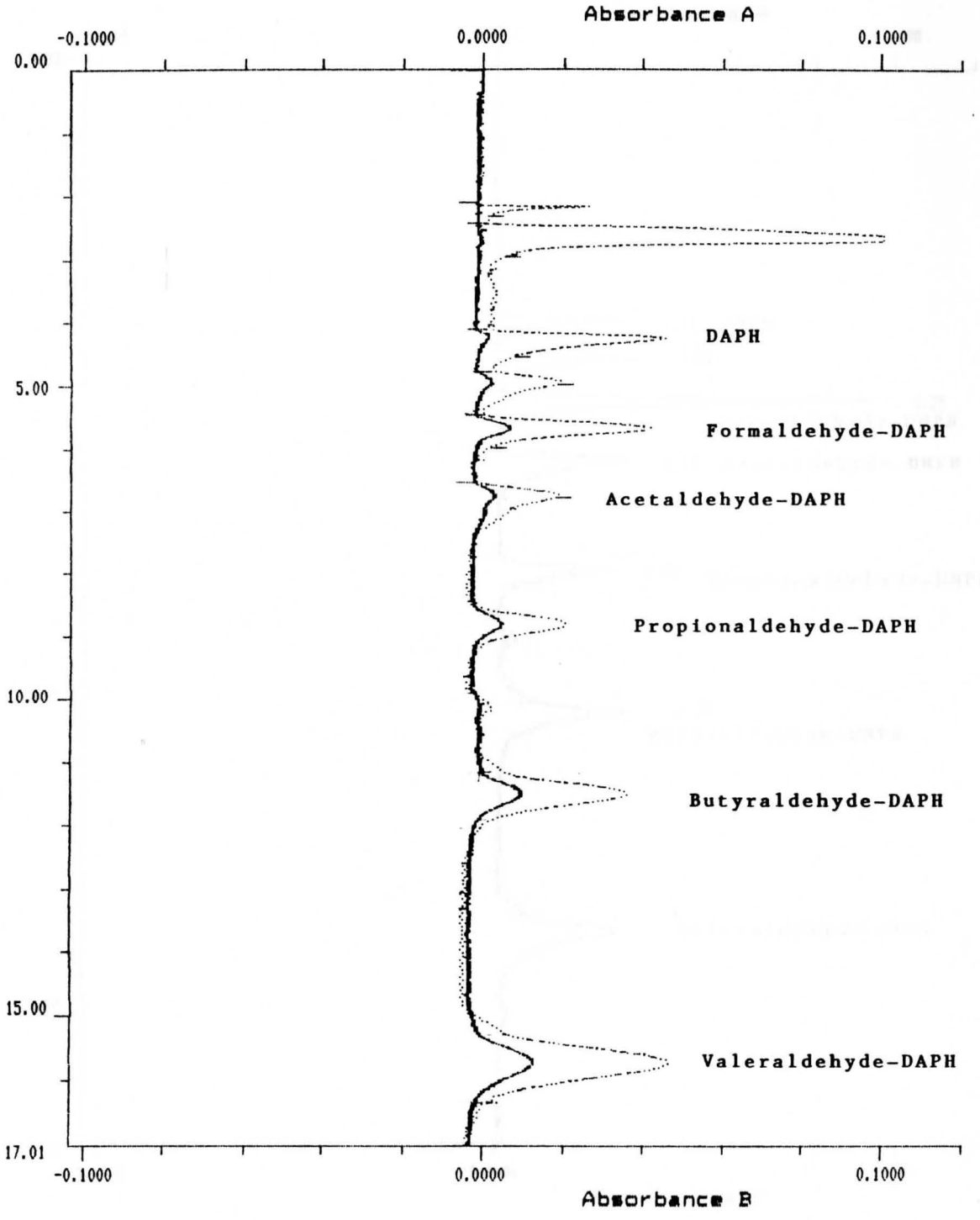


Figure 36: Chromatogram, Zinc Electrode, Potential Setting 50.0 Volts. Sample: 2,4-DNPH-aldehyde mixture derivative, 6.17 x 10^{-4} M. Sample 10 µL. Mobile Phase: 0.010 M sodium perchlorate monohydrate in 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. Voltage setting on power supply: 50.0 volts. UV detection at 345 nm (solid line), and at 212 nm (dashed line).

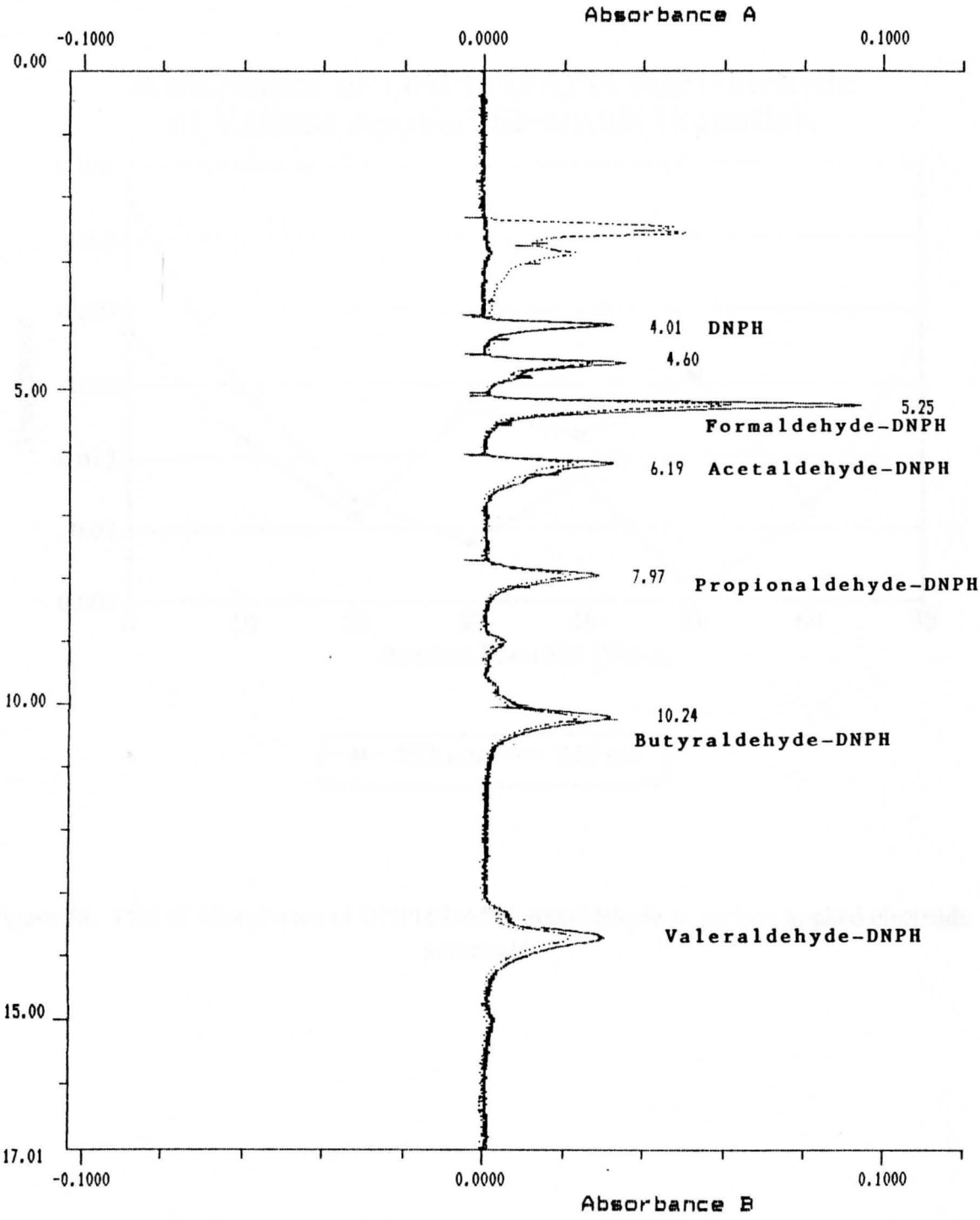


Figure 37: Chromatogram, Without Electrochemical Cell. Sample: 2,4-DNPH-aldehyde mixture derivative, 6.17 x 10^{-4} M. Sample size: 10.0 µL. Mobile phase: 0.010 M sodium perchlorate monohydrate in 55% acetonitrile-45% water. Flow rate: 1.00 mL/min. No electrochemical cell present. UV detection at 345 nm (solid line) and at 212 nm (dashed line).

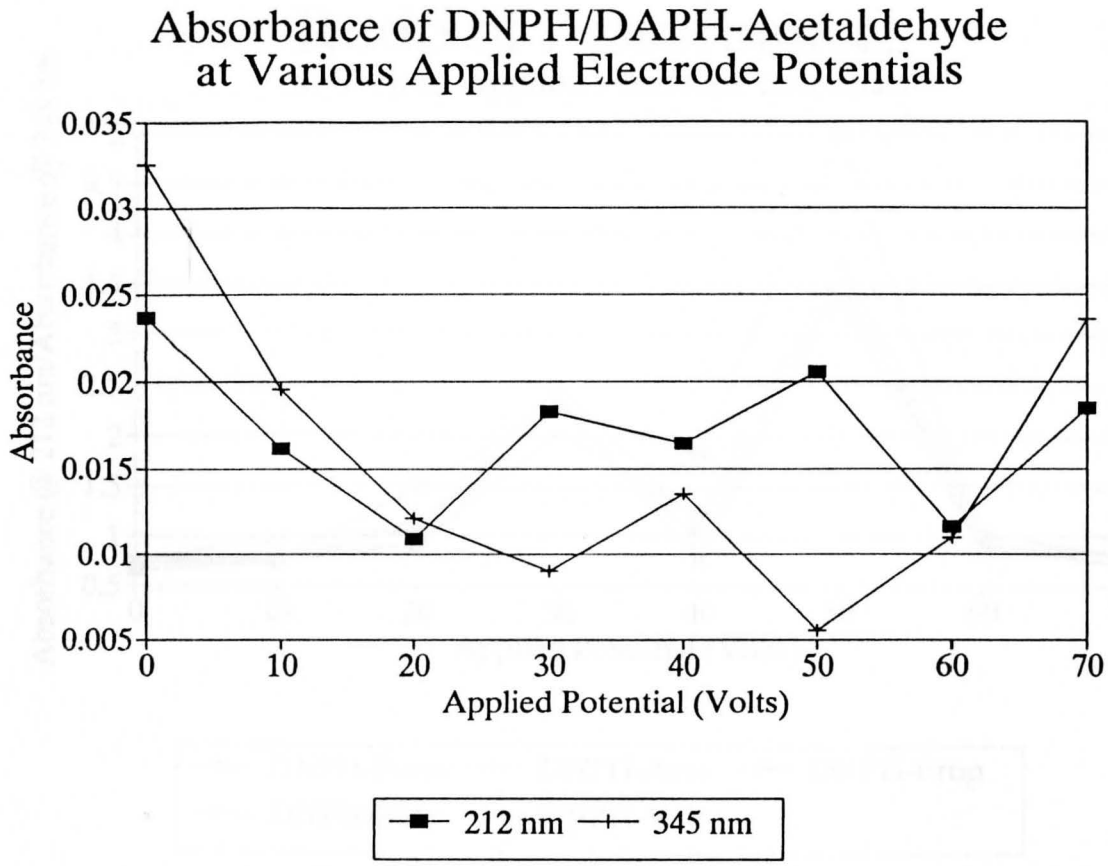


Figure 38. Plot of Absorbance of DNPH/DAPH-acetaldehyde at various applied electrode potentials.

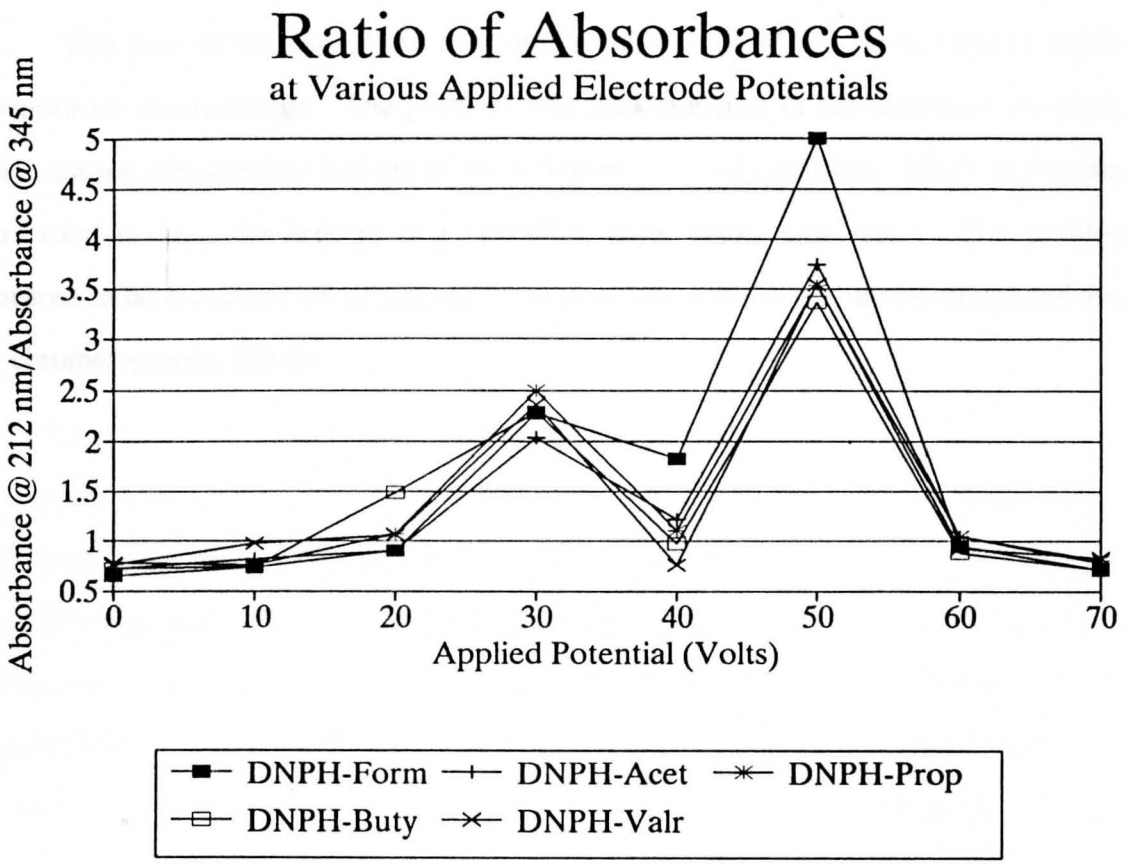


Figure 39: Plot of Absorbance Ratios at 212 nm/345 nm vs. Applied Potential in Volts from DNPH aldehyde derivatives.

Determination of Optimum Three-Electrode Construction

This part of the experiment was attempted, but could not be completed due to instrumental shortcomings. The problem was back-pressure in the reference electrode, which caused considerable leakage of the reference electrode solution. Many approaches were tried to stop the leakage (e.g., parafilm, tape, epoxy glue, etc.). The problem appeared to be a design flaw of the reference electrode and the way it was integrated into the chromatography system.

CHAPTER VII

CONCLUSIONS

Having completed this research topic, there are some conclusions that can be made. The first of these is that there is uncertainty in the fluorescamine reaction with the 2,4-DAPH aldehyde derivative. This compound apparently either does not readily form a fluorescent derivative or it may be that 2,4-DAPH is a compound that forms a non-fluorescent, yellow colored-chromophore, or perhaps even a non-fluorescent, non UV absorbing derivative. More likely is the possibility that the choice of fluorescamine as the derivatizing reagent may not be the appropriate one. Fluorescamine reacts well with primary amines. The structure of the post column derivative in this study was more like a derivative of aniline than the aliphatic amines for which fluorescamine is typically used. It would be appropriate to perform an investigation of fluorescence derivatization using an aniline reacting fluorogenic compound. The answer to these questions will require a bulk solution study of the fluorescence reactions using UV spectroscopy and fluorescence spectroscopy.

With regard to the electrochemistry several conclusions can be drawn. In the two electrode system, zinc served better than platinum as the appropriate electrode material, and it allowed for successful on-line electrochemical reduction of DNPH as evidenced by the increased absorbance at 212 nm over that of 345 nm.. There is still one problem that still remains with the electrochemical cell, that is better control of the potential on the working electrode. Implementation of a three electrode system is the next logical step for control of potential, but a newly designed reference electrode and possibly a newly designed electrode system must be developed before this will be successful.

Even given the failure of the fluorescence part of this study, there is evidence that the electrochemical reduction of the DNPH derivatives is a useful endeavor. Since the compounds absorb maximally at different wavelengths and have different absorption spectra, electrochemical reduction is an extremely useful tool for identification of aldehydes in complex chromatograms from real world samples. By simply looking at reduced versus unreduced chromatograms, and comparing peak absorbance diode array spectra one could distinguish the aldehydes from interfering compounds in the chromatogram. It would probably be advantageous to develop a series of absorbance ratios of various aldehyde derivatives to be used as a tool in identification. In addition, if one compares the absorbance at 212 nm for the 2,4-DAPH derivatives with the absorbance of the 2,4-DNPH derivatives, the amplitude is approximately equal so that detection limits for the 2,4-DAPH derivatives should be relatively the same as for the 2,4-DNPH derivatives unaffected. An excellent future study would be to utilize the electrochemical reactor for studies of aldehyde concentrations in air samples.

REFERENCES

(1) Hargis, Larry G. *Analytical Chemistry: Principles and Techniques.* pp. 494-547. Englewood Cliffs, NJ: Prentice-Hall, Inc., 1988.

(2) Lindsay, Sandie. *High Performance Liquid Chromatography: Analytical Chemistry By Open Learning*. New York: John Wiley & Sons. 1992.

(3) Stulik, K. and Pacakova, V. *Electroanalytic Measurements in Flowing Liquids.* Chichester, England: Ellis Horwood Limited. 1987.

(4) Frei, R. W., and Lawrence, J. F., (Editors). *Chemical Derivatization in Analytical Chemistry, Volume 1: Chromatography*. New York: Plenum Press. p. 127ff. 1981.

(5) Kyriacou, Demetrios K. *Basics of Electroorganic Synthesis.* New York: John Wiley & Sons. 1981.

(6) Allen, Milton J. *Organic Electrode Processes*. New York: Reinhold Publishing Corporation. 1958.

(7) Bard, Allen J., and Faulkner, Larry R. *Electrochemical Methods--Fundamentals and Applications.* New York: John Wiley & Sons Inc. 1980.

(8) Ramos, B. L., and Mike, J.H. *Microchem Journal* **47**, 33-40 (1993).

(9) Frei, R. F., Jansen, H., and Brinkman, U. A.Th. *Analytical Chemistry*. Vol. 57, No. 14, December 1985.

(10) Morrison, R. T., and Boyd, R. N. *Organic Chemistry, Fifth Edition*. Boston: Allyn and Bacon, Inc. p. 366. 1987.

(11) Silverstein, R. M., Bassler, G. C., and Morrill, T. C. *Spectrometric Identification of Organic Compounds, Fifth Edition* p. 289-294. New York: John Wiley & Sons, Inc., 1991.

(12) Bruan, Robert D. *Introduction To Instrumental Analysis.* p.153 ff. New York: Mc-Graw Hill Book Company 1987.

(13) Manahan, Stanley. *Toxicological Chemistry: A Guide to Toxic Substances in Chemistry.* p. 198-209. Chelsea, Michigan: Lewis Publishing, 1989.

(14) Barone, J. P. and Walter, T. H. *Waters Column.* 1-3. (Autumn 1991).

(15) Gutch, C. F. and Stoner, M. H. *Review of Hemodialysis for Nurses and Dialysis Personnel.* The C. V. Mosby Co. St. Louis, 1971.

(16) Houlgate, P. R.; Dhingra, K.S.; Nash, S. J. and Evans, W. H. *Analyst* **114**, 355 - 360. (1989).

(17) Committee on Aldehydes, Board of Toxicology and Environmental Hazards, National Research Council, "Formaldehyde and Other Aldehydes"; National Academy Press, Washington DC, 1981.

(18) Papa, L. J., and Turner, L. P. *J. Chromat. Sci.* **10**, 747-750 (1972).

(19) Selim, Sami. *Journal of Chromatography.* **136,** 271-277 (1977).

(20) Mansfield, C.T., Hodge, B. T., Hege, R. B., Hamlin, W. C. *J Chromat. Sci.* **15,** 301-302 (1977).

(21) Lam, S. K., and Margiasso, V. A. *Journal of Liq Chromatography.* **7,** 2643 (1984).

(22) Benassi, C. A., Semenzato, A. and Bettero, A. *Jounal of Chromatography* **464,** 387-393 (1989).

(23) Sigvardson, K. W. and Birks, J. W. *J. of Chromatography.* **316,** 507-518 (1984).

(24) Mike, J. H., Ramos, B. L., and Zupp, T. A. *Journal of Chromatography* **518,** 167-177 (1990) .

(25) Lawrence, J. F. *J. of Chromatographic Sci.* **17,** 147-151 (1979).

(26) Frei, R. W. and Lawrence, J. F. *J. of Chromatography.* **83,** 321-330 (1973).

(27) Uzu, S., Kanda, S., Imai, K., Nakashima, K., and Akiyama, S. *Analyst.* **115,** 1477-1482 (1990).

(28) Traore, F., Pianetti, G.A., Dallery, L., Tod, M., Chalom, J. Farinotti, R., Mahuzier, G. *Chromatographia.* **36,** 96-104 (1993).

(29) Iwata, T, Hirose, T., Nakamura, M., and Yamaguchi, M. *Analyst.* **118,** 517-519 (1993).

(30) Weigele, M., DeBernardo, S. L., Tengi, J. P., Leimgruber, W. *Journal of Amer. Chem. Soc.* 94:16. August 9, 1972.

(31) Felix, A. M. and Terkelsen, G. *Anal. Biochem.* **60,** 78-87 (1974).

(32) Udefriend, S., Stein, S., Bohlen, P., Dairman, W. *Science.* **178,** 871-872 (1972).

(33) Stein, S., Bohlen, P., Stone, J., Dairman, W., and Udenfriend, S. *Arch. of Biochem and Biophysics.* **155,** 202-212 (1973).

(34) Bohlen, P., Stein, S., Dairman, W., and Udenfriend. *Arch. of Biochem and Biophys.* **155,** 213-220 (1973).

(35) Sogawa, K., and Takahashi, K. *J. Biochem.* **83,** 1783-1787 (1978).

(36) Samejima, k., Kawase, M., Sakamoto, S., Okada, M. and Endo, Y. *Anal. Biochem.* **76,** 392-406 (1976).

(37) Frei, R. W., Michel, L., and Santi, W. *J. of Chromatography.* **142,** 261-270 (1977).