THE DETERMINATION OF ORGANIC NITROGEN COMPOUNDS IN WATER BY HIGH SPEED LIQUID CHROMATOGRAPHY

By

JERRY DALE CAPLINGER II

Bachelor of Science

Central State University

Edmond, Oklahoma

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THE DETERMINATION OF ORGANIC NITROGEN COMPOUNDS IN WATER BY HIGH SPEED LIQUID CHROMATOGRAPHY

Thesis Approved:

Louis P. Varga
Thesis Adviser

Larry L. Scarlett II

W. F. Gaudy Jr.

N N Durham
Dean of the Graduate College

PREFACE

The objective of this study was to develop a liquid chromatographic system capable of separating organic nitrogen compounds present in natural water. Standards and laboratory algae cultures were used to test the separation capabilities.

The study was conducted under the guidance of Dr. Louis P. Varga, who served as major adviser. The other members of the advisory committee were Dr. H. L. Gearhart and Dr. A. F. Gaudy. Their contributions were instrumental in the completion of this project.

Special thanks goes to Mike Pierce for his help and friendship. Finally I would like to thank my wife for her support and patience during this investigation.

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CHAPTER I

INTRODUCTION

Water pollution has become the concern of many scientists in recent years. The diversity of their fields is due primarily to the large number and wide variety of chemical and biochemical interactions which continuously occur in natural water systems. Of these the nitrogen cycle is among the most interesting, the most complex, and least understood from a quantitative viewpoint. A simplified scheme of the nitrogen cycle is displayed in Figure 1 (1). Although there are many other elements, compounds, and cycles required for biosynthesis, nitrogen and phosphorous have long been considered limiting nutrients for primary production. Naturally occuring organic nitrogen compounds consist primarily of amino and amide nitrogen. Traces of some heterocyclic compounds such as pyrmidines and purines have been found (2). The presence of even low concentrations of these organic nitrogen compounds affect not only the color, odor, and taste of natural water, but also the chemical equilibrium necessary for biological growth of microorganisms.

Chlorine has also been found to react with organics containing nitrogen to produce compounds which are toxic to acquatic life and which are potentially harmful to human life (3).

The primary problem for the analyst investigating amino acids, amines, pyrmidines, and purines is that they occur naturally only in

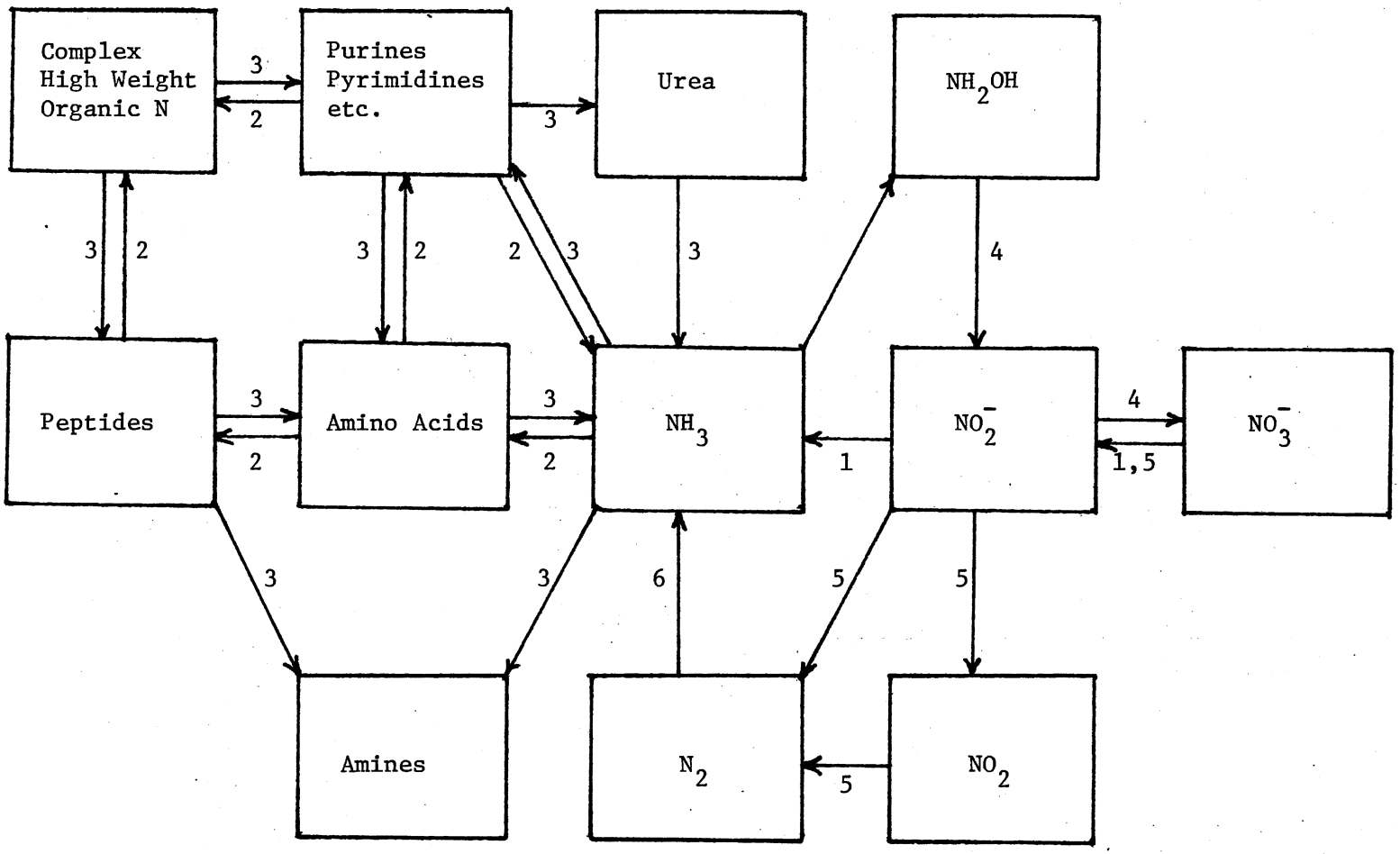

Figure 1. Simplified Nitrogen Cycle Showing Main Molecular Transformations: 1. Nitrate Assimilation, 2. Ammonia Assimilation, 3. Ammonification, 4. Nitrification, 5. Denitrification, 6. Nitrogen Fixation (1)

trace amounts. To detect these minute concentrations, elaborate concentration techniques, which can introduce error in the analysis, must be devised (4).

The objective of this study was to develop a simplified method for the separation and detection of trace organic nitrogen compounds which involve a minimum number of concentration procedures. The system chosen for this work has high performance liquid chromatography optimized for the separation and detection of organic nitrogen compounds known to be present in natural water systems.

CHAPTER II

LITERATURE SURVEY FOR THE DETERMINATION OF ORGANIC NITROGEN COMPOUNDS

Introduction

The concentration of dissolved organic nitrogen compounds in natural and waste water systems is found to be at the trace level. However, even with low concentrations, a large number of different forms have been found to be present and many more expected. The determination of trace organics containing nitrogen and other dissolved organics has required numerous analytical methods. All methods involving the qualitative or quantitative analysis of organics in water have three analytical techniques in common: 1.) concentration of the samples in such a way as not to alter or degrade the constituents, 2.) separation of the constituents into their appropriate classes and individual compounds, and 3.) the detection of each component by the most sensitive and most specific methods possible. Illustration of these three techniques will be given following a short summary of nitrogen compound sources and sinks in lake and stream water.

Sources and Sinks

The concentration and forms of nitrogen in a lake at any given time are a function of the input rates, the interconversion reaction

occuring within the lake, and the rate of loss by way of outflow, denitrification, and sediment deposition. Nitrogen input into lakes is generally considered to occur faster than the output, thus producing accumulation of nitrogen compounds in the lake water and sediments. Erdman (5) detected several amines and amino acids in the Gulf of Mexico sediments that were three million years old.

A thorough review of the inorganic nitrogen sources of lakes in general was given by Feth (6). Brezonik (1) presents a detailed discussion of nitrogen sources and transformations in natural water. A summary of these sources and sinks for the nitrogen budget in a lake are shown in Table I (1).

Concentration

Numerous concentration techniques have been used in trace water analysis. Freeze drying, distillation, precipitation, adsorption, and solvent extraction are the most commonly used and all give reasonable recovery rates (7).

Distillation is one of the oldest methods, but is still practiced by many investigators. Vacuum distillation was used by Peterson (8) in 1925 to enrich samples from a Wisconsin lake for sequential analysis. The excretion of amino acids from a laboratory culture of blue-green algae was studied by Stewart (9). Vacuum distillation was used to concentrate these trace organics. Chau (10) described some of the advantages of rotary film evaporators over precipitation methods in the analysis of amino acids in the Irish Sea. Primary effluent from waste water treatment plants can also be concentrated by vacuum distillation as was shown by Katz.

TABLE I

SOURCES AND SINKS FOR THE NITROGEN BUDGET OF A LAKE (1)

Sources	Sinks
Airborne	Effluent loss
Rainwater	
Aerosols and dust	Groundwater recharge
Leaves and miscellaneous debris	
	Fish harvest
Surface	
Agricultural (cropland) and drainage	Weed harvest
Animal waste runoff	
Marsh drainage	Insect emergence
Runoff from uncultivated and forest land	
Urban storm water runoff	Volatilization (of NH_3)
Domestic waste effluents	
Industrial waste effluent	Evaporation (aerosol formation from surface soam)
Waste from boating activities	
	Denitrification
Underground	
Natural groundwater	Sediment deposition of detritus
Subsurface agricultural and urban drainage	
Subsurface drainage from septic tanks near lake shore	Sorption of ammonia onto sediments
In situ	
Nitrogen fixation	
Sediment leaching	

Coprecipitation of organic nitrogen compounds has been found useful in fresh and salt water analysis. Park (12) tried ferric chloride in the determination of seventeen amino acids in the Gulf of Mexico. Four years later Tatsumoto (13) performed similar work in the Gulf using mercuric chloride. The hydrolized precipitate was further concentrated by aid of vacuum distillation.

The sediment samples taken from the Gulf of Mexico by Erdman (5) were solubilized and the amino acid content increased by freeze drying. Katz (11) used vacuum distillation for his initial concentration, followed by freeze drying the remaining solute.

One technique which allows the use of various solvents is solvent extraction. Aniline and pyrine were found in effluent from coal tar plants after extraction with diethyl ether. Bark (14) also detected other substituted aniline compounds in the waste water. Diethyl ether was found useful by Caruso (15) for extraction of trace organics from river basins. Litchfield (16) made "dansyl" derivatives of eighteen amino acids and extracted them with ether. Rotary vacuum distillation aided in the completion of the concentration procedure. Methylene chloride was used by Mieure (17) to extract small amounts of organics added to tap water. Rotary evaporation of California seawater to dryness, and then dissolving of the precipitate in ethanol, was tried by Degens (18). Palmork (19) found that amino acids would react with 2,4-dinitro-1-fluorobenzene to produce an ether extractable derivative.

Carbon adsorption in trace water analysis was reviewed by Ryckman (20,21) in two separate papers. It's recovery capabilities were compared with those of liquid-liquid extraction in the first paper.

Jeffery (22) compared electrodialysis, carbon adsorption, ion exchange, solvent extraction, coprecipitation, and vacuum distillation as to their percent of recovery of amines, amino acids, and other organic nitrogen compounds. Samples from the Gulf of Mexico were concentrated with each of the above techniques.

Recently, Amberlite microreticular resins have been shown to have great promise in pollution analysis. Junk (23) has studied recovery capabilities of ten organic nitrogen compounds found in water. Burnham (24) used the resins for concentrating pesticides in well water.

Separation

The organic nitrogen constituents of water range from the acidic amino acids to the basic amines. The acid and basic characteristics of these compounds make them susceptible to separation by cation and anion exchange chromatography. Paper, thin-layer, and column chromatography have all been successfully used in the analysis of soluble amines and other carbon and nitrogen pollutants. Several other separation techniques have been used, but these three chromatographic methods are the most popular and successful.

Paper chromatography is probably the simplest of the three techniques, and was used the most frequently by earlier investigators. Individual component quantification by paper and thin-layer chromatography was difficult and often only qualitative identification was performed. Degens (18) was able to use two dimensional paper chromatography to separate seventeen amino acids with concentration of 16 to 125 micrograms per liter from California sea water. Indoles were

analyzed for, but none were found. Paper chromatography was also used by Jeffery (22) to compare the efficiencies of five concentration procedures used on the Gulf of Mexico. Several amines and amino acids were detected. Stewart (9) also used paper chromatography to measure amino acid flux of laboratory algae cultures. Parsons (25) reported the identification of several amino acids in oceanic detritus by paper chromatography.

Thin-layer chromatography has found little direct use in water analysis. It, however, was used by Chau (10) on the Irish Sea for the determination of eleven amino compounds with concentrations of 2 to 16 parts per million. Palmork (19) devised a scheme in which the amino acids in sea water could be reacted with 2,4-dinitro-1-fluorobenzene, and then extracted with ether before separation with circular thin-layer chromatography. Amines and amino acids have also been found to form "dansyl" derivatives which are extractable from fresh or salt water. Litchfield (16) was capable of separating 17 amino acids with this method, but four different solvent systems were necessary.

Protein hydrolysates have been separated with gravity flow of different pH buffers over a column packed with cation exchange resin. Seventeen organic compounds were found in the Gulf of Mexico and near British Honduras with aid of this method used by Tatsumoto (13). Urea and several members of the uracil family were quantitatively detected in primary effluent by Katz (11) with high resolution anion exchange chromatography. Ertinghausen (26) has developed a fully automatic system for the analysis of amino acids in 63 minutes by ion exchange chromatography.

Gas chromatography appears to be the most popular separation technique with nearly half the investigators using this method. Burnham (27) successfully separated trace organics including aniline, phenyldiamine, and p-nitrophenol in concentrations of .37 to 15 parts per billion with gas chromatography. These compounds were concentrated from Iowa water wells with XAD resin. Aniline and substituted aniline compounds were present in coke-oven effluent when analyzed with gas chromatography. Bark (14) was able to detect them in concentrations of 1.2 to 16.5 mg/l with a flame ionization detector. Mieure (17) separated nitrophenol, chloroaniline, chloronitrobenzene, and numerous other amines and amides present in waste water or added to tap water. Gas chromatography was the technique chosen by Park (12) for the semi-quantitative determination of seventeen amino acids in the Gulf of Mexico.

Autoanalyzers were useful in measuring the flux rate of amino acids in the New York River. Hobbie (28) noted the presence of seventeen amino acids after standard addition of carbon-14.

Detection

Total bound nitrogen is often determined in conjunction with individual component identification. Micro Kjeldahl procedures are the most popular and reliable for dissolved organic nitrogen concentration (29). This method is based on the fact that organic nitrogen can be converted into ammonium sulfate when oxidized with hot H_2SO_4 and a catalyst.

The infrared, ultraviolet, visible, refractive index, and fluorescence characteristics of various organic nitrogen compounds have

been used in their detection. Katz (11) was able to detect 77 trace organics in primary waste with ultraviolet spectroscopy. Refractive index was one of the detectors chosen by Junk (23) for detection of organics in real water which included 10 nitrogen compounds. Riley (30) used photometry to investigate the uptake of amino acids by XAD resins, only to find they were not retained. Many nitrogen compounds cannot be directly detected by photometric methods and must be reacted with light absorbing compounds. Ninhydrin has found extensive use for amino acid determination. Ertinghausen (31) used ninhydrin to detect 17 amino acids eluded from an ion exchange column. Ninhydrin was also the indicator Degens (18) used in his paper chromatographic separation of amino acids. Recently a new reagent has been synthesized which reacts almost instantaneously with primary amines, peptides, proteins, and amino acids to produce a fluorescent product which will allow analysis in the picomole range (32).

Carbon-14 counting was performed by Jeffery (22) to quantitatively detect nitrogen compounds which were not susceptible to ninhydrin indicator. Carbon-14 counting has also been used to measure the algae excretion rate of amino acids. The chromatographic mobilities of 13 tritium labeled and 23 radiocarbon labeled amino acids relative to their unlabeled forms were measured on ion exchange columns by Klein (33).

Mass spectroscopy has been heavily relied upon as a quantitative and qualitative tool by several investigators. This is largely due to its direct linkup capabilities with gas chromatographs. Junk's (23) investigation of 77 organics, including 10 nitrogen compounds, which might be present in natural water was performed largely with gas

chromatography/mass spectrometry (GCMS). Mieure (17) used both flame ionization and mass spectrometry as detectors for his gas column effluents.

Litchfield (34) used microbiological assay for the determination of the distribution of adenine, uracil, and threonine in the Gulf of Mexico. Although no threonine was found, adenine was found in ten samples and uracil in two.

Summary

Of the organic compounds present in aquatic systems amino acids have been found to be among the most concentrated. Because of these high concentrations, compared to other nitrogen compounds, many investigators have focused only upon their determination. Very little work has been done on the analysis of other families of organic nitrogen compounds in natural water. A method is thus needed that will allow determination of some of these trace compounds and still be sensitive to the amino acids.

CHAPTER III

PRINCIPLES, THEORY, AND TECHNIQUES OF LIQUID COLUMN CHROMATOGRAPHY

History

Separation of components by chromatography was first discovered by Twsett (35) in 1906. It was 35 years later when liquid partition chromatography was developed by Martin and Synge (36) and reported in their classic 1941 paper. Paper chromatography and gas chromatography were introduced about 1952, and have since received more attention then liquid chromatography because of their speed and ease of separation. Gas chromatography was also found to have more versatile and sensitive detectors than its liquid counterpart.

Difficulties with pumps, packing, detectors, and columns have retarded the advancement of liquid chromatography as an analytical tool until recent years. In 1969 Kirkland (37) was primarily responsible for the development of a stable high performance controlled porosity column packing. Miniature flow-through cells have greatly increased the sensitivity of liquid chromatography but their further advancement is still needed (38). Precision bore stainless steel columns with polished inner surfaces and high pressure pumps have allowed the use of smaller and more efficient packing with diameters of 3 to 5 μ (39).

Liquid Chromatography

Liquid chromatography is divided into four classes according to the nature of the stationary phase which dictates the mechanism of separation. The differences between these classes is often not rigid and may overlap. The four classes of liquid chromatography (LC) are liquid-solid (adsorption) chromatography, liquid-liquid (partition) chromatography, ion exchange, and gel permeation and gel filtration (exclusion) chromatography. Partition and ion exchange were both used in the experimental determination that follows, and will thus receive primary focus in this chapter.

Liquid-liquid chromatography (LLC) is performed by passing a mobile phase across a solid stationary support coated with a liquid phase. Separation results because of the difference in solubilities of the solutes in the stationary and mobile phases. Polar mixtures are resolved by normal partition chromatography which requires a polar stationary phase and a less polar mobile phase. Reverse phase partition uses a polar mobile phase and a less polar stationary phase to separate nonpolar mixtures.

In ion exchange chromatography, the column is packed with an insoluble solid stationary support, containing groups which by electrostatic forces can reversibly exchange either cation or anions present in a mobile wash solution. The type of functional group attached to the superficially porous beads dictates if the packing will be anion or cation exchange resin and also determined the strength of the resin.

Theory

The theory of liquid chromatography is essentially the same as other chromatographic methods except for the differences in some of the terms due to the variation in technique. The degree to which two components are separated is defined as the resolution (R_s) and is determined by:

$$R_s = 2 \frac{(t_{R1} - t_{R2})}{(W_1 - W_2)} \quad [1]$$

where t_{R1} and t_{R2} are the retention times of two retained components and W_1 and W_2 are the widths of the bases of their respective peaks as defined in Figure 2.

For high speed liquid chromatography if R_s is less than .8 separation is considered to be unsatisfactory. An R_s greater than 1 would represent baseline separation.

The number of theoretical plates (N) in a liquid chromatographic column is a measure of the column efficiency and can be calculated by:

$$N = 16 \frac{(t_{R1})^1}{(W_1)^2} = 16 \frac{(t_{R1})^2}{(2\, W_{h\frac{1}{2}})^2} \quad [2]$$

where $W_{h\frac{1}{2}}$ is the width at half height. The height of a theoretical plate can then be found by dividing the number of theoretical plates by the length of the column as in equation 3:

$$HETP = \frac{(N)}{(L)} \quad [3]$$

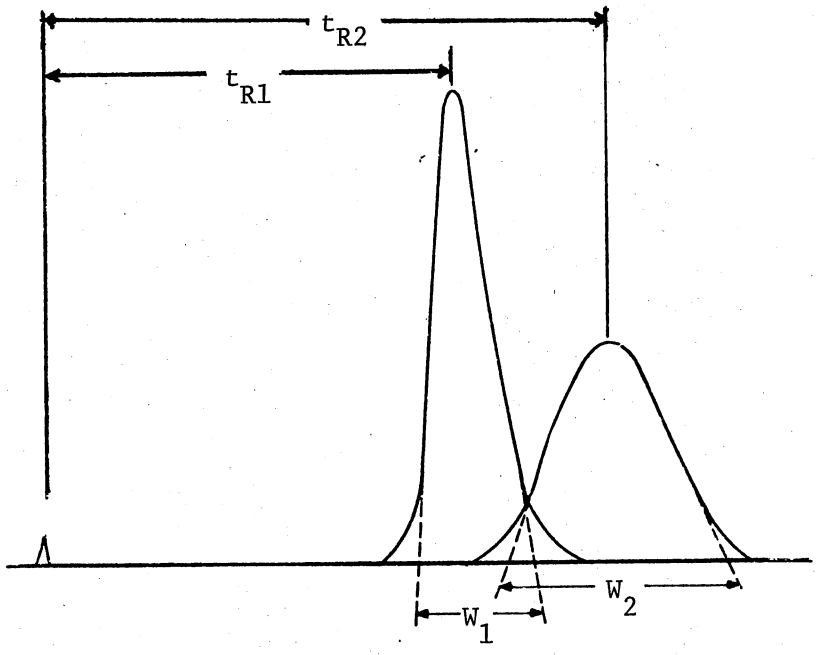

Figure 2. Example Chromatogram

The relative separation of different components is termed the column selectivity (a) and is defined as:

$$a = \frac{(t_{R2} - t_m)}{(t_{R1} - t_m)} = \frac{(t'_{R2})}{(t'_{R1})} = \frac{(K_2)}{(K_1)} \qquad [4]$$

where t'_{R1} and t'_{R2} are the adjusted retention times of components 1 and 2 respectively, K_1 and K_2 the distribution coefficients of components 1 and 2 respectively, and t_m the retention time of an unretained component.

The retention volume of unretained components (V_m) and the volume of the stationary phase can be calculated from the flow velocity and other column parameters. The elution volume can then be defined in terms of these two quantities and the distribution coefficient (K) by equation 4.

$$V_R = V_m - (K)(V_s) \qquad [5]$$

Equation 6 defines the capacity factor k' and shows how it can be determined directly from the retention times or retention volumes:

$$k' = K\frac{(V_s)}{(V_m)} = \frac{(t_R - t_m)}{t_m} = \frac{(V_R - V_m)}{V_m} \qquad [6]$$

Peak areas are calculated by equation 7:

$$A = (W_{h\frac{1}{2}})(h) \qquad [7]$$

where A is the area, h is the height.

CHAPTER IV

EXPERIMENTAL ACTIVITY

Introduction

The experimental procedure has been divided into three sections, but are all interrelated. The first procedure was to make improvements on an existing liquid chromatograph which included the installation of a new detector. A diagram of this chromatograph is shown in Figure 3 (40). The next step involved the choice of a chromatographic system which would separate a mixture of reagent grade nitrogen compounds known to be present in natural water. A field study, which required the collection of samples from Lake Carl Blackwell, concentration of the trace organics, and their separation and detection, was the final step performed in this experiment.

The Liquid Chromatograph System

Solvent Reservoirs

Two types of solvent reservoirs were used. The first type was glass and delivered the solvent directly from the bottom of the reservoir into the inlet of the pump. This type reservoir was hard to handle when changing solvents, and air bubbles were often trapped in the lines connecting the reservoir and pump. This reservoir was soon replaced with a 500 ml flask in which the solvent was delivered to the

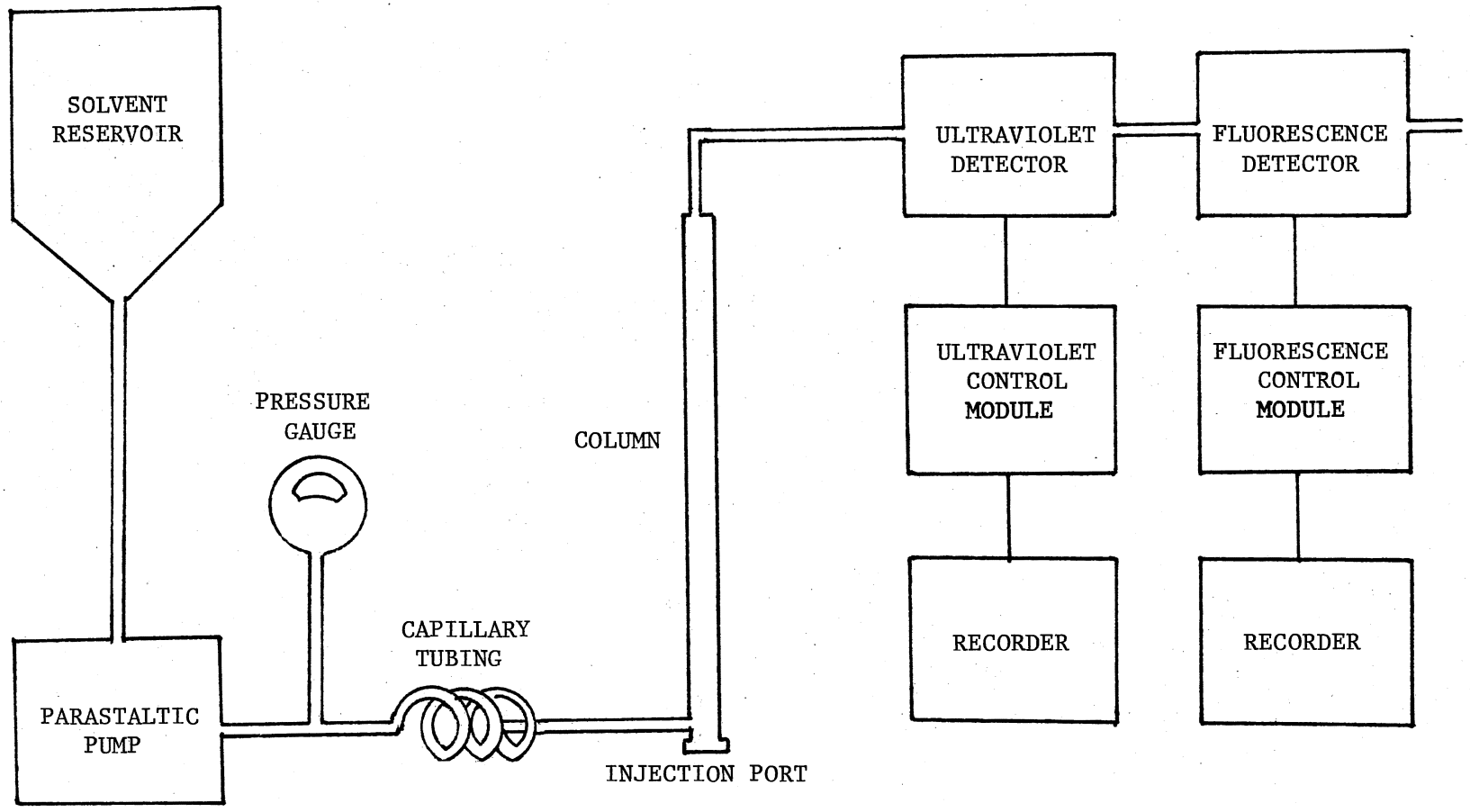

Figure 3. Diagram of a Dual Detector Liquid Chromatograph

pump by siphonage. The tubing connecting the pump and reservoir was converted to stainless steel and glass to prevent leaching of organics from plastic tubing when using organic solvents in the reservoir. The delivery tubes were designed small enough to hold the siphon while solvents were being changed. The mobile phase was degassed in the same flasks that would later be reservoirs.

Pump

Constant volume and constant pressure pumps that can deliver pressures up to 6000 psi are commercially available. The type used in this chromatograph was constant volume Milton Roy pump (Model Number 196-0042-059). The volume delivered can be preset by changing the stroke length of the piston diaphragm. The problems involved with this type of pumps are that pressure surges develop with each stroke of the pump, and that the pump chamber volume is too large to allow recycle or easy gradient elution. The maximum pressure for this pump was 1000 psi which is sufficient for most packing material with diameters of 30 microns or larger.

Pressure Gauge

A United States Gauge (Model Number 29528-1) with a range of 0 to 1000 psi was used to monitor the on line pressure. The gauge was inserted parallel to the solvent flow with aid of a tee. Capillary tubing was used to connect the gauge to the injection port. The large volume gauge and small diameter tubing act similar to an electrical RC filter and help reduce pressure surges produced by the reciprocating pump.

Columns and Fittings

Stainless steel precision bore tubing of various lengths was used for columns. The tubing had a 1/8 inch outside diameter and a 2 millimeter inside diameter. Stainless steel adaptors were used to connect the column and detector. These adaptors contained a porous metal frit, and an inert porous filter also was needed to keep the packing in the column.

Injection Port

Sample introduction into the liquid chromatograph was made with syringe injections. Both stop flow and on line injections were made during the course of this experiment. The injection port was designed such that the sample could be introduced downstream from where the solvent flow enters the injection port. Part of the injection port was packed with glass wool to reduce band broadening. The sample was injected into the glass wool and then swept onto the column. Several septum materials were tried, but Applied Science Laboratories W-9 proved to be the most inert and allow the most injections before failure. When injecting under pressure, care had to be taken to hold the syringe plunger in tightly or glass wool would be forced into the syringe barrel. A Hamilton high pressure syringe (Number 88200) was used for all injections when the flow was not stopped. Reproducible high pressure injections are harder to obtain than stop flow injections.

Detectors

Dual series detectors were incorporated to allow greater detection capabilities than a single detector. The first detector was a Varian Aerograph (Model Number 02-001428-03) with a mercury discharge lamp emitting ultraviolet light at 254 nm. The cell shown in Figure 4 has a cell volume of 8 microliters. This detector was equipped with a reference cell which was kept full of the mobile phase being used. Due to a fault in the cell design air bubbles were often trapped in the upper part of the sample and reference cells. These air bubbles could be detected with the photometer on the UV controller and they were very troublesome to dislodge. This detector was connected directly to the column outlet by capillary tubing and zero dead volume fittings.

The second detector was a Farrand Fluorometer (Model Number 104224B Series 190). This detector contained a quartz cell which is connected to the Ultraviolet detector by plastic flexible tubing. This instrument did not contain a reference cell. The signal obtained from the emission of the solvent was electrically zeroed. The recorder had to be rezeroed each time the excitation or emission wavelength monochrometer was changed. A high intensity Zenon arc lamp was used as the source for the sample excitation. Fluorescence was measured at a right angle to the incident beam. The sample cell contained an internal volume of 30 microliters and a path-length of 5 millimeters. Because many compounds absorb and emit light at different wavelength values, fluorescence can be used as a semiqualitative instrument. Obtaining maximum sensitivity from the Zenon lamp required adjustment of the lamp orientation after each ignition.

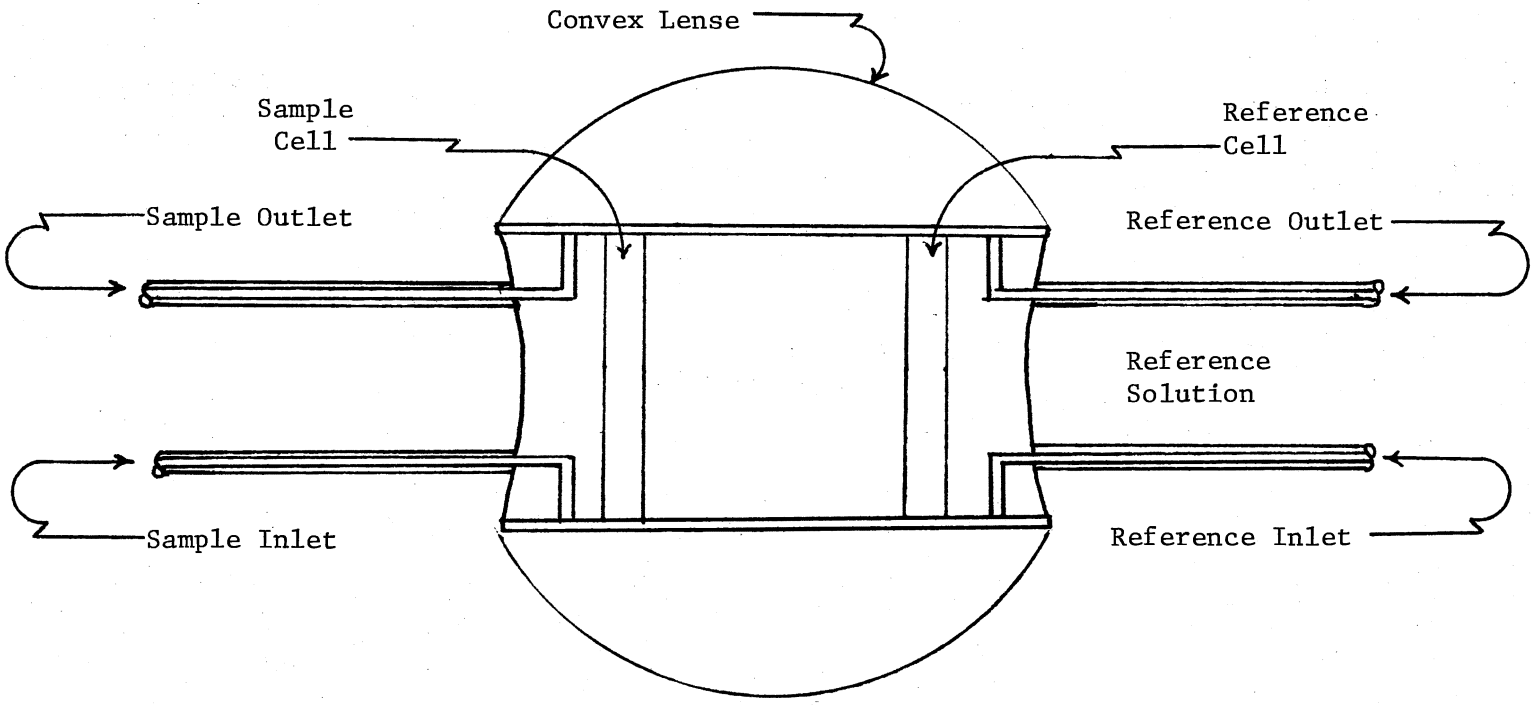

Figure 4. Diagram of an Ultraviolet Flow-Through Cell

Sample Solvent

A distinction should be made between the term sample solvent and mobile phase because they may or may not be the same solution. The solvent and mobile phase must, however, be soluble in each other. If they are not the same, a large solvent peak will likely be present. Most of the nitrogen compounds tried in this experiment were moderately polar and were only soluble in solvents with high polarities. The same solvent and mobile phase were used with the ion exchange packing, but an ethanol-water mixture solvent and acetonitrile mobile phase were required for the reverse phase separation with a Permaphase ETH column. Water proved to be a good solvent for all the nitrogen compounds in this investigation.

Column Supports

It can easily be seen that the organic nitrogen compounds separated in this study were not all from one class. Because of this class difference their chemical characteristics varied, as would be expected. The only feature they had in common besides containing nitrogen was that they were all aromatic and were all moderately polar. These three chemical similarities were the reason for selection of the stationary supports which were investigated. Three different packings were tried before reasonable separation of the components could be obtained.

The first stationary support tried was a chemically bonded type known as Permaphase ETH (41). This packing is a porous layer bead of 37-50 microns in diameter with a Zipax spherical base and an ether

functional group. The ether group gives the packing a high polarity which allows it to be used with low polarity solvents for normal phase partition chromatography. It also can be used with high polarity solvents for reverse phase partition chromatography. The amino acids proved to be too insoluble in low polarity hydrocarbons to allow the use of normal phase. Numerous mobile phases were used in conjunction with the Permaphase ETH packing in the reverse phase mode. These included ethanol-water in various mixtures ranging from 100% ethanol to 100% water. Solutions of ethanol-acetonitrile were also investigated, but proved to be much less satisfactory than the ethanol-water mixtures. Other mobile phases tried included various percent solutions of acetonitrile-isopropyl ether. These mixtures also proved to be less satisfactory than the ethanol-water solutions. The ETH column used for these experiments were dry packed and was two feet in length.

Since it can easily be seen from Chapter II that ion exchange has been used extensively for the separation of organic compounds containing nitrogen, this was the next packing to be tried. Strong Anion Exchange (SAX), a one percent lauryl methylacylate polymer produced by Dupont, was chosen (42,43). Zipax beads are the support particles on which the quaternary ammonium ($-NR_3^+$) group and polymer will bond to produce 25-37 μ packing particles. The functional group was expected to interact differently with the acidic and basic compounds. Buffered solutions of water were the mobile phases chosen. Retention times on SAX are theoretically not affected by pH, but changes were observed and attributed to the weak exchange group on the sample components. A pH of range 6.6 to 7.8 was found to give optimum resolution of the most components. Several lengths of column were tested to

obtain the best resolution in the shortest amount of time. A 3 foot and a 2 foot column connected by a porous frit were used in the final lakewater analysis.

The last column and solvent system selected was the nonpolar bonded packing Permaphase ODS (44,45). This was a Zipax support bonded to one percent octadecylsilane by ether linkage to form 25-36 μ packing. The suggested mobile phase mixtures of ethanol-water were tried with almost no success. Short retention times suggested that the nitrogen compounds were too polar to be partitioned by the nonpolar ODS packing even with 100% water.

The solvent and mobile phase purity were as follows: distilled and deionized water, 95 percent technical grade ethanol, reagent grade isopropyl ether, and nano grade acetonitrile. Problems with degassing, leakage, corrosion, and detector stability were more prevelant with the organic solvents.

Organic Nitrogen Compounds

The nitrogen compounds chosen as standards for this experiment were selected from amino acids, pyrimidines, purines, amines, acids, and indoles. Three amino acids, tyrosine, tryptophan, and phenylalanine, were chosen because they were the only ones which fluoresce. The ratio of their fluorescence intensities are 5:100:1 respectively. The other amino acid chosen was histidine. Uracil, a pyrimidine, and adenine, a purine, were selected to represent nucleic acid hydrolysis products. Both possessed good ultraviolet absorption characteristics. The aromatic amine aniline was found to have measurable absorption and fluorescent capabilities and was used to check for instrument

problems. Indole was found not to be fluorescent but could be seen by the 254 nm detector. Uric acid has been detected in lakes and was included in the list of organics selected for separation.

All standards were prepared using the purest available reagents. Tryptophan purity was in question because of the shape of the LC peak it gave. The samples were kept at 4°C to prevent the action of bacteria on the organic nutrients. Peak distortion from bacteria growth could be detected within 48 hours after sample preparation. Sodium hydroxide was added as needed to water solvent to aid in the solubilization of the sample components.

Preconcentration Procedures

The degree of preconcentration desired for this investigation was 100 fold. This value was selected because it was sufficient to concentrate aromatic organics in concentrations of about 50 ppb or more in natural water, sufficiently enough to be detected photometrically. This 100 fold concentration could be accomplished with one liter of sample thus not requiring special containers and apparatus. Four concentration procedures (ion exchange, liquid-liquid extraction, distillation, and freeze drying) were investigated using one liter of a 1 ppm solution prepared from the stock 100 ppm solution. The percentage recovery of the standard solution could then be calculated and used to evaluate the usefulness of the procedure. The best two techniques were then used on the natural water systems.

Liquid-Liquid Extraction

Liquid-liquid extraction was the first procedure used. The one liter of 1 ppm solution was first acidified to pH 2 with HCl (conc.) and then extracted with three 25 ml volumes of chloroform. Next, enough NaOH (conc.) was added to the aqueous phase to produce a pH of 10. The standard mixture was then extracted with three more 25 ml volumes of chloroform.

The first 75 ml of chloroform were then back extracted with two five ml portions of water at pH 10 and the second 75 ml portion was extracted into 10 ml of water at pH 2. This extraction was repeated on a second and third standard mixture using ether and tributylphosphate respectively as the organic phases.

Ion Exchange

The cation exchange resin used was DOWEX 50W-X4. The column was glass, 30 cm in length, and 15 mm in diameter. The resin was washed with 200 ml of 6 N HCl and then rinsed with five 200 ml portions of distilled water. Preconcentration was performed by passing one liter of the standard mixture across a column followed by stripping of the retained organics with several successive 10 ml portions of 6 N HCl. Each 10 ml sample was collected and treated separately.

The anion exchange resin used was DOWEX 1-X8. The same column was used but the washing and stripping of the resin was accomplished using 6 N NaOH in place of the HCl in the procedure above.

Distillation

Vacuum distillation was tried first but proved to be troublesome and unsuccessful and was abandoned. Steam distillation was also tried but it too was hard to control.

One liter of the standard 1 ppm solution was distilled until the temperature of the distillate rose above 100°C. The sample remaining (about 100 ml) was then transfered to a smaller distillation flask and distilled to 10 ml. The temperature of the distillate never rose above 120°C.

Freeze Drying

Freeze drying proved to be the longest yet easiest and probably most efficient procedure. In this procedure the one liter of sample was frozen in a three liter flask and the sample was then dried on a lyophilizer. The residue was then dissolved in 10.00 ml of distilled water containing one drop of HCl to aid in the solubilization.

It should be noted that a residue of humic material remained after distillation or freeze drying. This material was found to be very insoluble in acids, bases, or organic solvents. Centrifuging of the sample was necessary in order to obtain an injectable supernate.

Applications

After a successful liquid chromatographic system for the separation of the standard mixtures had been achieved several experiments were performed to test the usefulness of it to natural water systems.

The first experiment involved water taken from Lake Carl Blackwell during October 1975. A second set of samples were taken from laboratory algae cultures. In both cases the water samples were filtered through 5 μ millipore filters to remove the solids and then concentrated by one of the methods described above.

Urine samples were also taken and injected, without any prior concentration, as a further application of the ion exchange column to biological fluids.

CHAPTER V

DISCUSSION AND RESULTS

Standard Mixtures

The reason for the selection of the organic nitrogen compounds used in the standard mixture is given in Chapter IV. This wide variety of nitrogen compounds was used to find a liquid chromatographic column and mobile phase system capable of separating naturally occuring organics found in lake water. After little success with polar and nonpolar packings and solvents strong anion exchange resin (SAX) was selected as the column support. Single component and multi-component injections were made at various pH's and column lengths until the best resolution in the shortest amount of time was obtained.

Of the 9 components in the standard mixture 7 of them could be detected in concentrations of 100 ppm or less by either the ultraviolet or fluorescence detector. It can be seen from Figure 5 that aniline, uracil, uric acid, adenine, and tryptophan had sufficiently high absorption characteristics to be detected by the 254 nm detector. Indole could also be detected but was not included in most of the chromatograms because of its long retention time (about two hours). Sequential analysis of the mixture by fluorescence indicated a response to aniline and tryptophan at an excitation wavelength of 280 nm and an emission wavelength of 340 nm, Figure 6. Aniline and tryptophan were

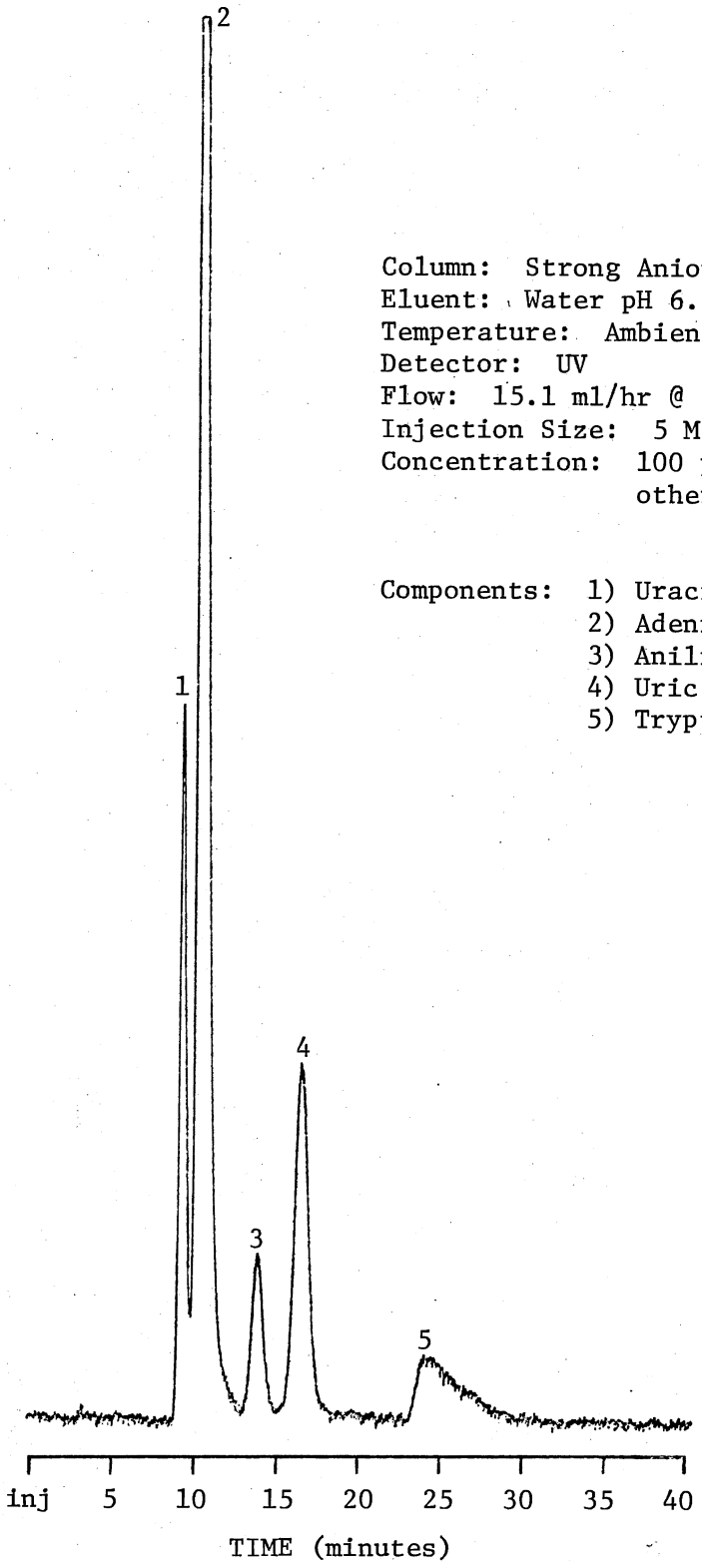

Figure 5. Separation of the Standard Mixture (UV Detector)

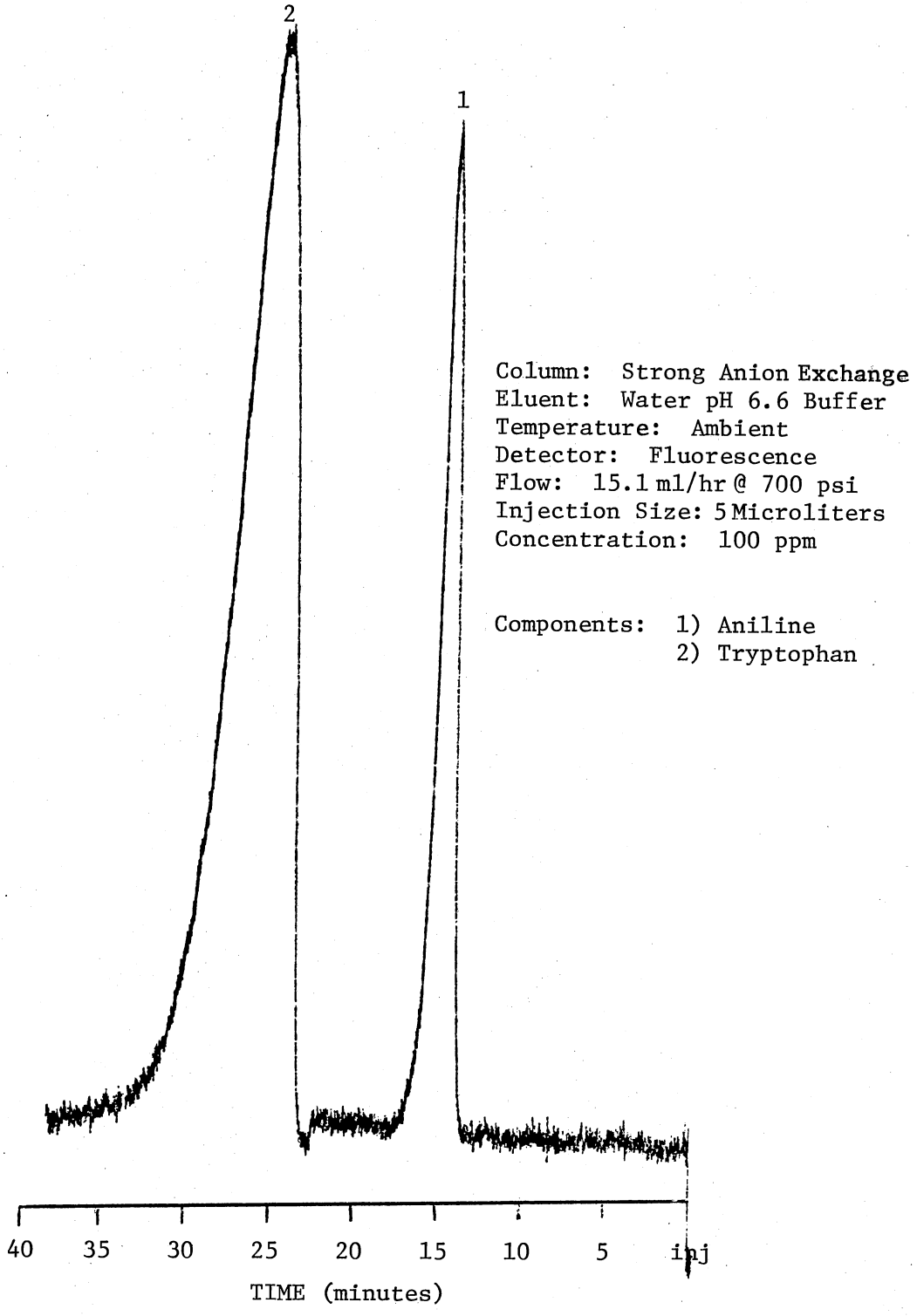

Figure 6. Separation of the Standard Mixture (Fluorescence Detector)

both found to be more sensitive to the fluorescence detector. Excitation and emission wavelengths of 275 and 310 respectively were used to detect tyrosine. No response to histidine or phenylalanine was recorded by either detector.

Fluorescence chromatograms obtained from the injection of four concentrations of aniline is displayed in Figure 7. This procedure was repeated with each of the 7 other components except indole. The more sensitive detector was used for quantification. Figure 8 is a summary of the peak areas for the standard curves of six components.

HETP for high performance liquid chromatography should be between .3 and 1.0 mm. Table II shows that two of the compounds are within this range. The HETP's were calculated with equation 2 and 3 using uncorrected retention times so as to be comparable with literature values.

Evaluation of the Concentration Procedures

Liquid-Liquid Extraction

Direct liquid-liquid extraction proved difficult because the organic compounds present in natural water can be acidic, basic or neutral. Single aqueous organic extractions would have been preferred but would require the extraction of one liter of water by just 10 ml of the organic phase to obtain a 100 fold concentration step. The high volume ratios and the insolubility of the organic phase in the mobile phase of the liquid chromatograph eliminated one step extractions. The two step method, discussed in Chapter IV, proved to be ineffective also when applied to the standards with only aniline being extracted.

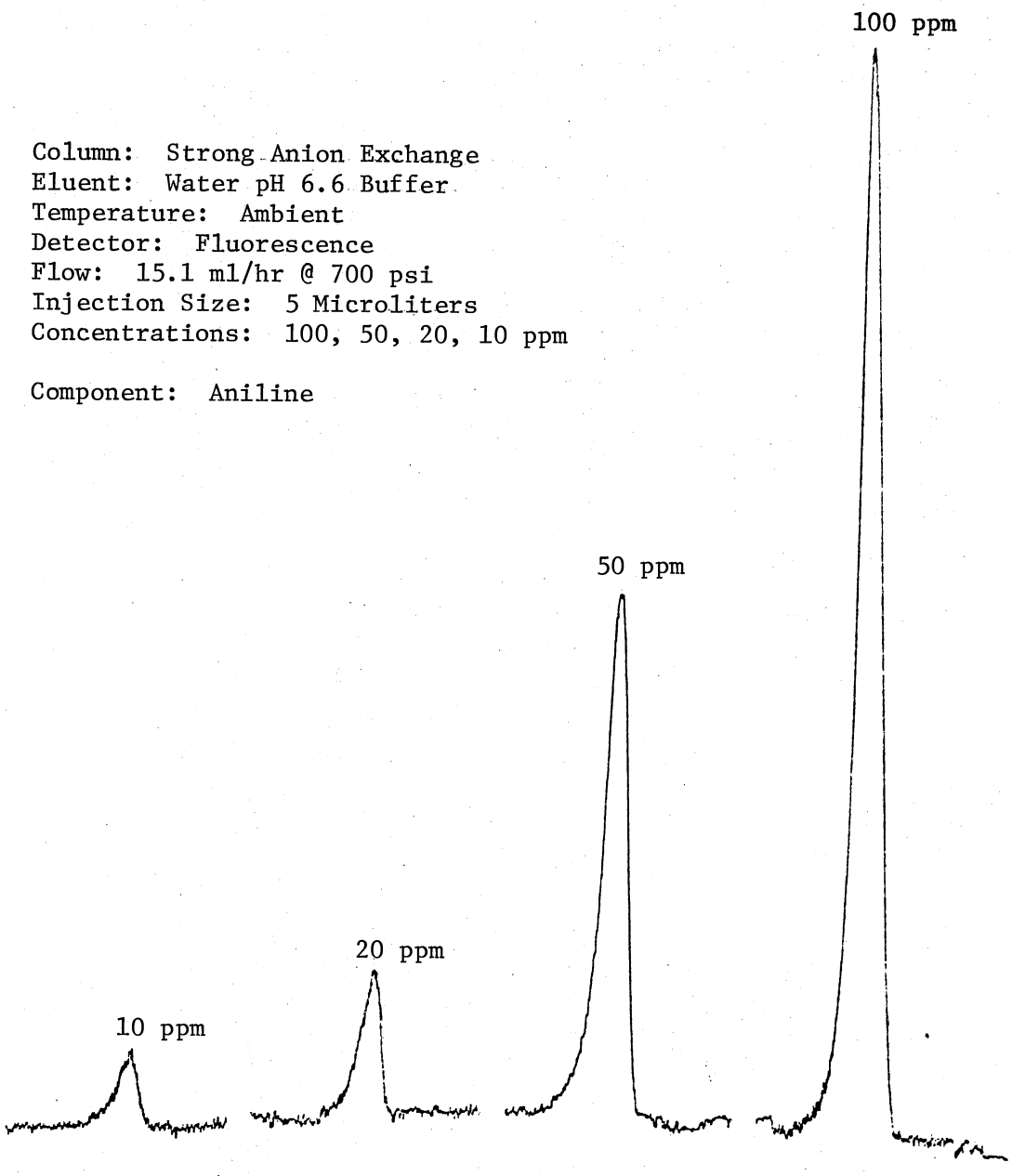

Figure 7. Four Injections of Different Aniline Concentrations

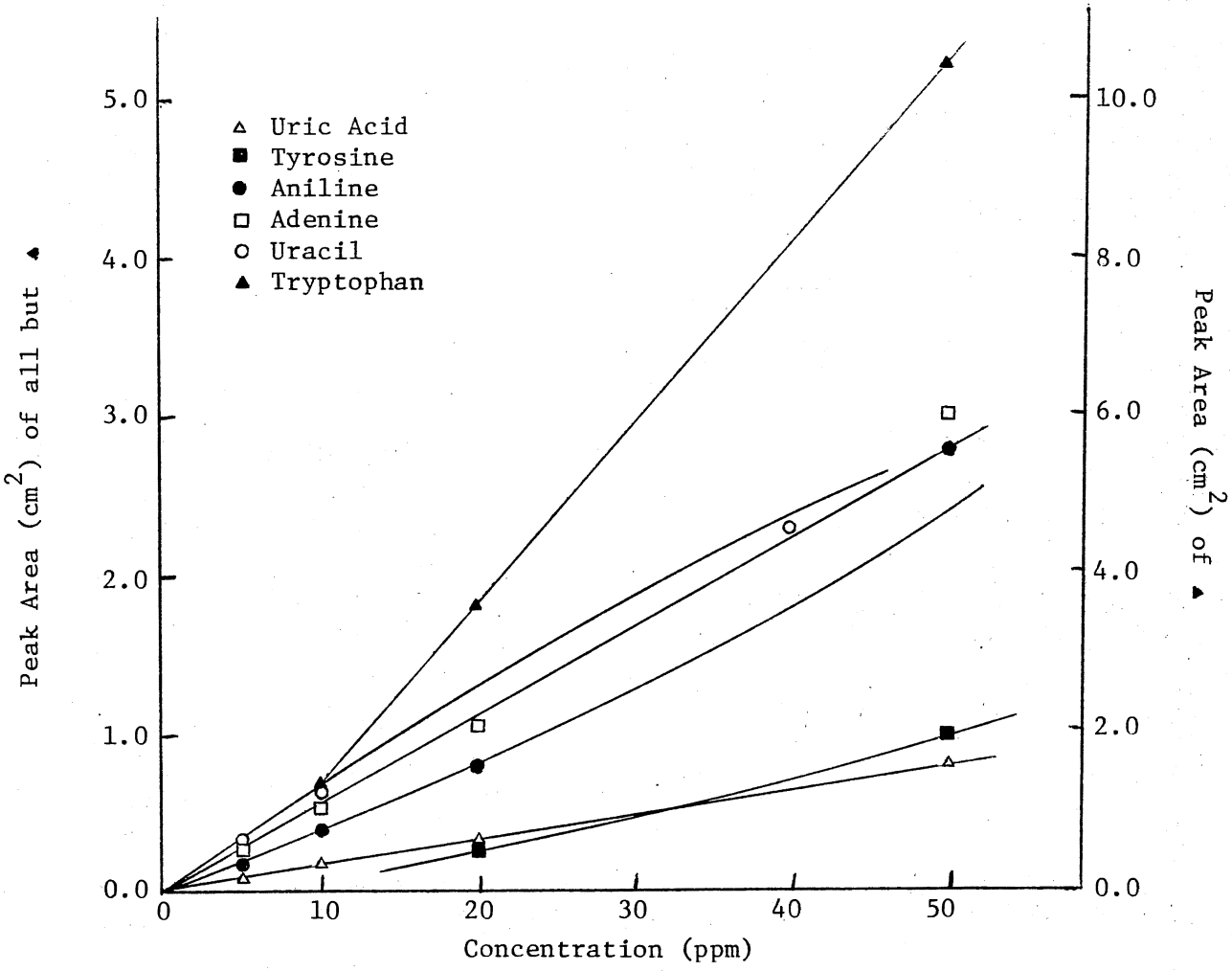

Figure 8. Standard Curves for Six Compounds by Peak Area (Equation 7)

TABLE II

BASIC CHROMATOGRAPHIC PARAMETERS FOR THE SEPARATION OF ORGANIC NITROGEN COMPOUNDS BY ION EXCHANGE

Solute	t_R (cm)	W_R (cm)	N	HETP* (mm)	k'**	a***	R_s †
Uracil	1.97 (7.75 min)	.20	1536	.80	.09	--	--
Adenine	2.27 (8.94 min)	.52	305	4.0	.26	2.8	1.87
Aniline	2.93 (11.54 min)	.29	1633	.74	.627	6.7	5.73
Uric Acid	3.51 (13.82 min)	.46	932	1.30	.95	10	6.82
Tyrosine‡	4.37 (17.20 min)	.77	515	2.4	1.43	14	9.29
Tryptophan‡	23.88 (94.02 min)	5.76	275	4.42	12.3	130	7.78

*HETP calculated using a 1215 mm column.

**Calculated using t_m = 1.80 cm (7.09 min).

***Relative to uracil.

†Relative to the preceding compound.

‡Data taken from fluorescence detector.

Ion Exchange

The large elution volumes necessary to remove the organics retained by the column were found to be troublesome. Injection of each fraction was time consuming and detracted from the analytical technique. None of the standards were recovered from the cation exchange column. 65 percent recovery of uracil was obtained using the anion exchange column.

Distillation

Due to the large amount of heat involved during distillation a loss of volatile organics and the molecular transformation of organic compounds can occur. The chromatogram for the standard solution implied no alterations had occurred but some may be present in the case of the algae samples. As can be seen by Figure 9 uracil and adenine were almost completely recovered while no trace of the other components present was found.

Freeze Drying

Freeze drying was found to be much more efficient than the other three techniques in terms of the number and percentage of standards recovered. Figure 10, however, shows that no aniline was recovered as was expected, due to its volatility. Only about 20% of the uric acid was recovered compared to 59% of the tryptophan. Uracil and adenine were almost totally recovered.

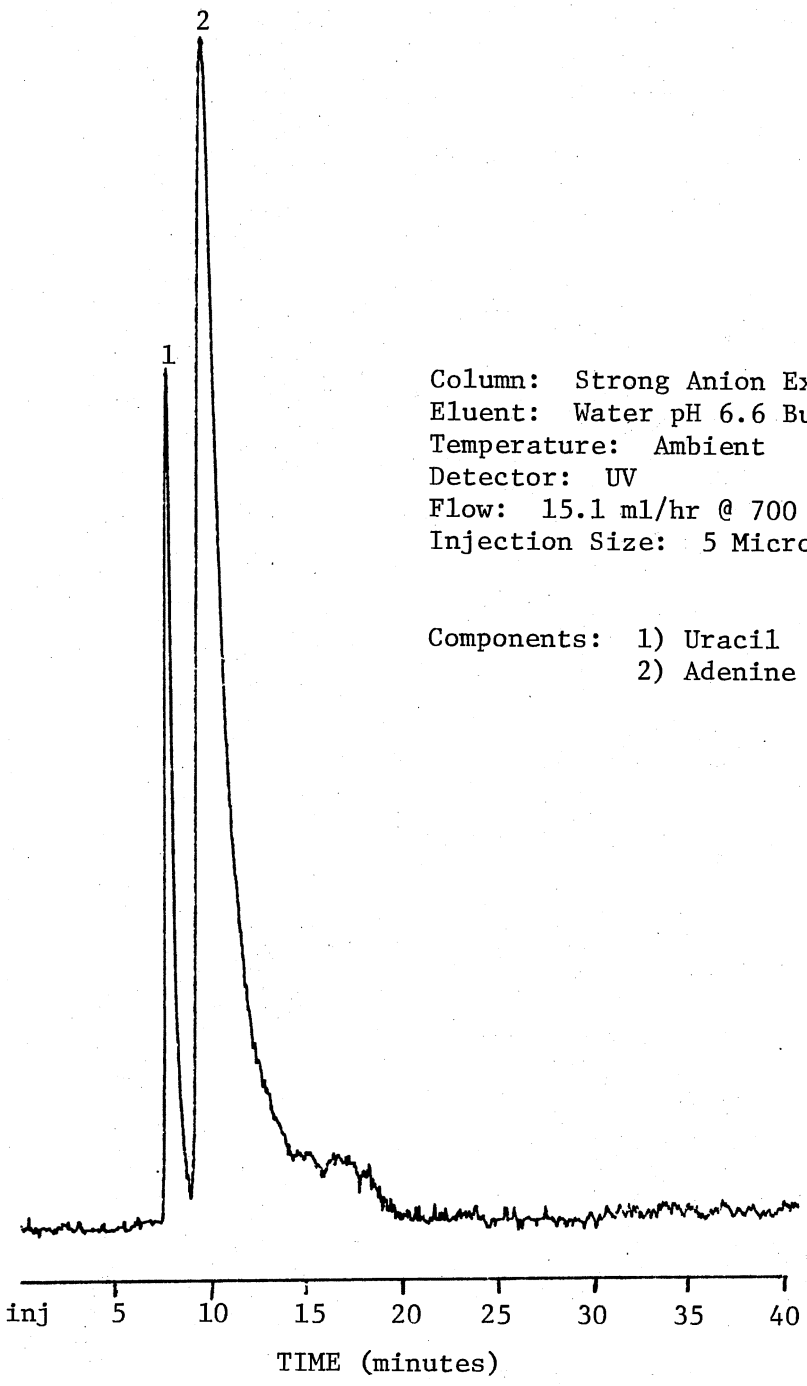

Figure 9. Components Recovered From Standard Mixture After Concentrating by Distillation

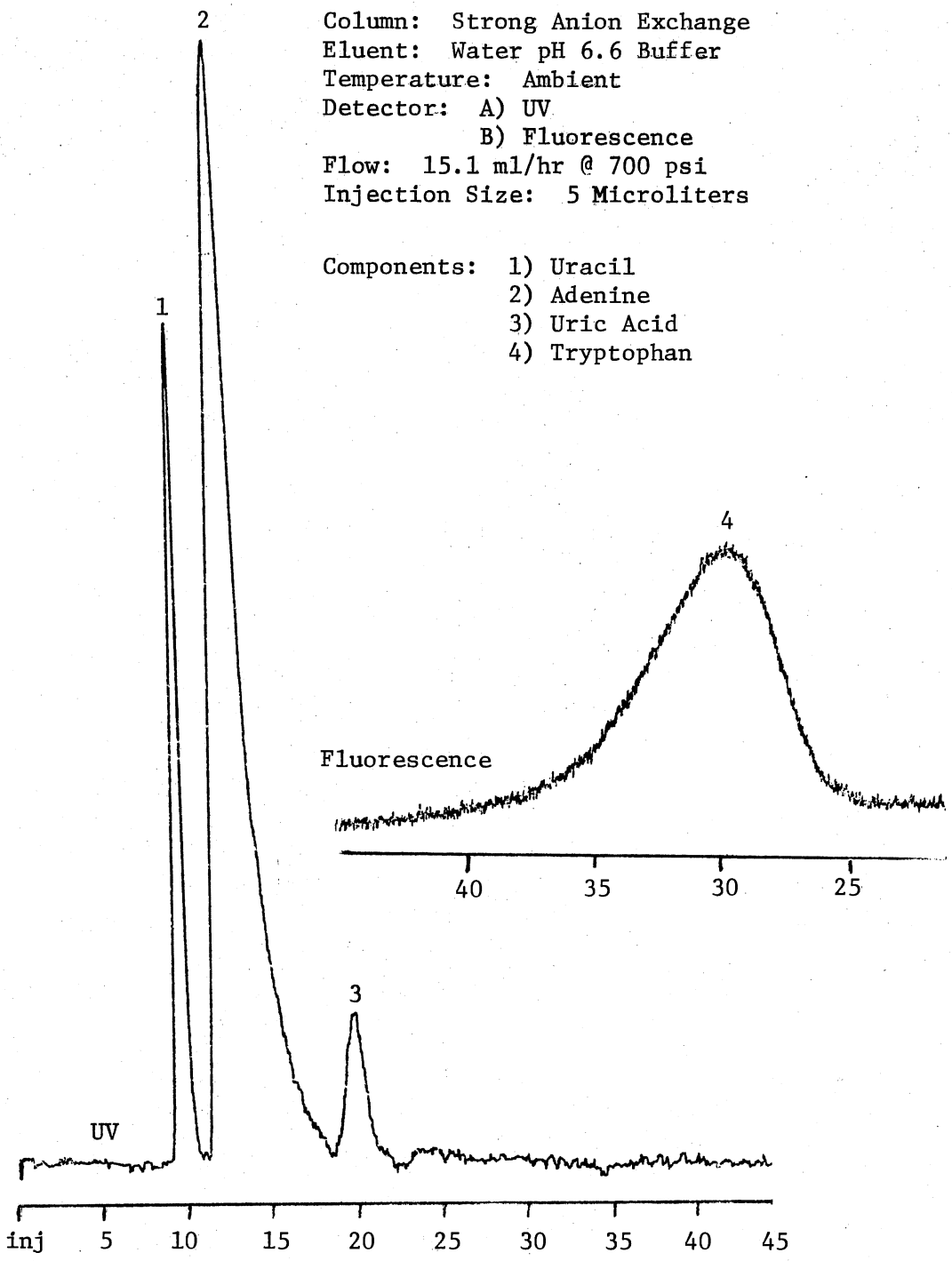

Figure 10. Components Recovered From Standard Mixture After Concentration by Freeze Drying

Summary

Salt residues remaining after freeze drying or distillation of the lake or algae cultures were removed by the addition of HCl in small amounts. The chromatograms of the concentrate from the laboratory algae cultures demonstrated that the number of organic compounds obtained by freeze drying or distillation is about the same, Figures 11 and 12 respectively. Table III gives a summary of the percentage of each compound recovered.

Lake Carl Blackwell Samples

Two samples were taken from the lake, filtered and freeze dried. The liquid chromatogram obtained gave only one small peak on the ultraviolet detector and none on the fluorescence detector. This low level of organics could be accounted for by the fact that the samples were not taken until late in the fall when only small amounts of photoplankton were present. The samples could not be taken in the summer when higher levels are expected because the liquid chromatographic separation of the standards had not been completed.

Laboratory Algae Cultures

Algae cultures were grown in the laboratory on only inorganic nutrients so that any soluble organic compounds detected were produced from the synthesis and excretion from biological life or that released from the breakdown of cell walls of dead organisms. These algae cultures were used primarily because of the low levels of organics present in Lake Carl Blackwell.

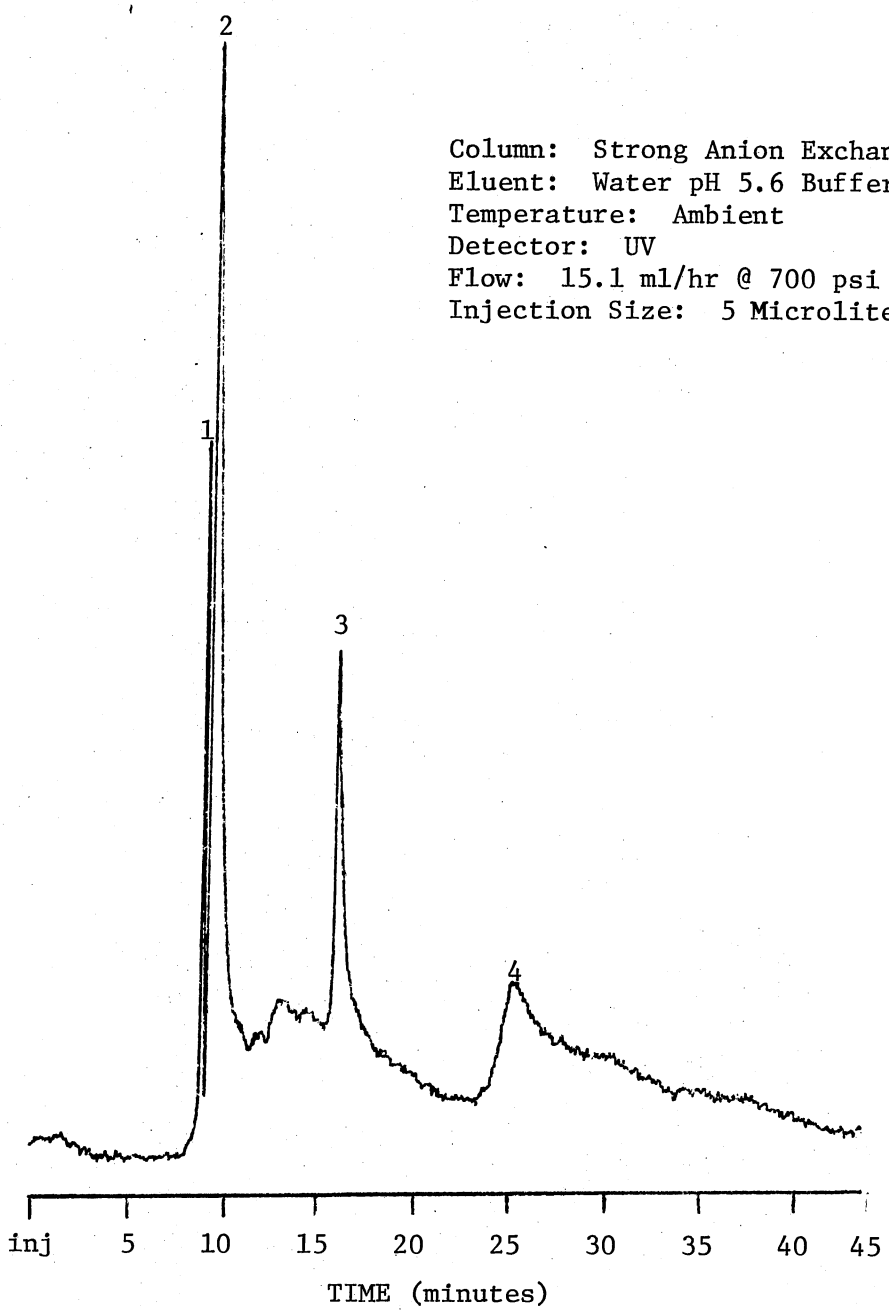

Figure 11. Chromatogram of Algae Culture Solution Concentrated by Freeze Drying

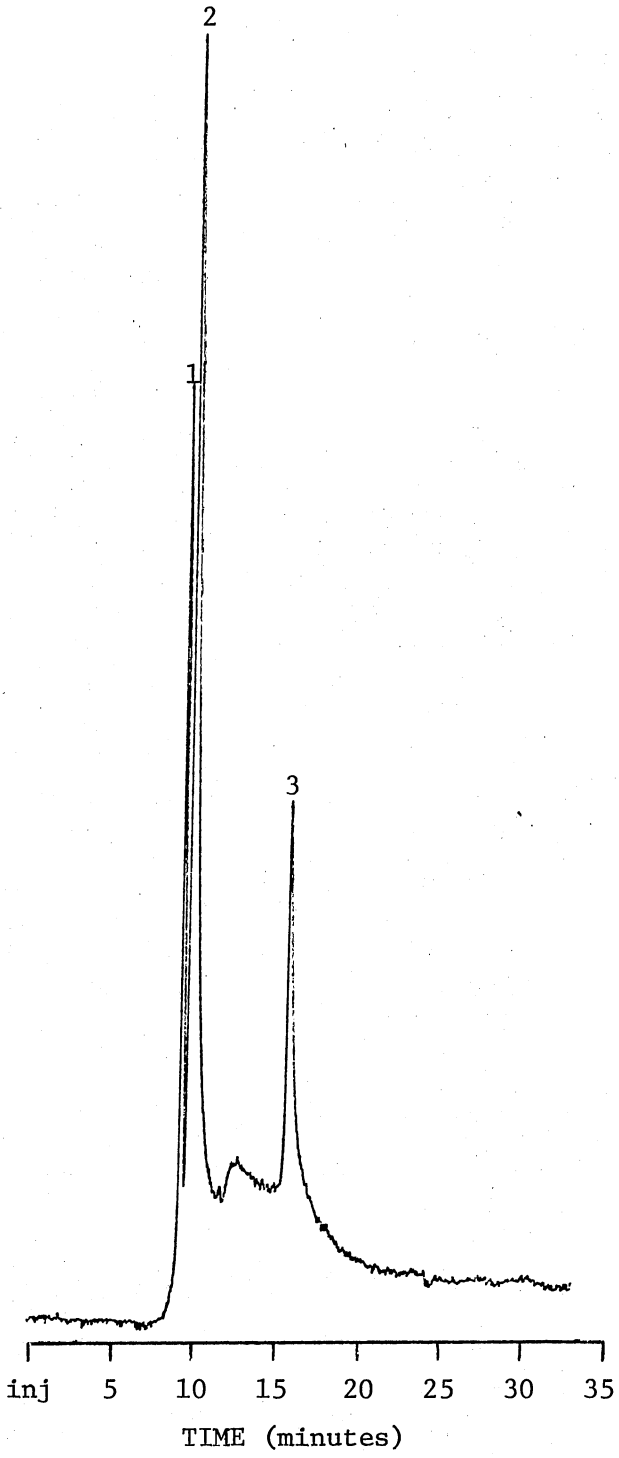

Figure 12. Chromatogram of Algae Culture Solution Concentrated by Distillation

TABLE III

SUMMARY OF RECOVERY DATA FOR SEVERAL
ANALYTICAL CONCENTRATION METHODS

Solute	Distillation	Freeze Drying	Chloroform Extraction	Ether Extraction	TBP Extraction	Anion Exchange
Aniline	-	-	45%	-	15.5	-
Uracil	91.3%	107%	-	-	-	65%
Uric Acid	-	18%	-	-	-	-
Adenine	85.5%	102%	-	-	-	-
Tryptophan	-	58.7%	-	-	-	-

Filtration of the algae cultures gave a supernate which was concentrated by freeze drying and distillation. A 200 fold concentration of the supernate was necessary to produce reasonable detector response. This was obtained by reducing the final volume from 10 ml to 5 ml. Figure 11 was obtained from the freeze drying concentration technique while Figure 12 was obtained from the distillation method. It was necessary to change the pH to 5.6 to obtain the best separation of these samples. Mass spectral identification of the peak components was impossible without further concentration of the peak eluent.

Standard addition was performed using uracil and uric acid because their elution times near those of the three major peaks in the algae samples. Equal volumes of the standard and algae solutions were mixed and then injected. The retention times of components number 2 and 3 in Figure 11 were identical to those of uracil and uric acid respectively. This gave tentative identification of these components by retention times.

Biological Applications

To further show the applicability of this chromatographic system a sample of urine was injected, Figure 13. No concentration procedure was used and the chromatogram still contained eight peaks with only a one microliter injection compared to 5 for previous injections.

Summary

The first major objectives of this study were to modify the existing liquid chromatograph to produce a more efficient and versatile

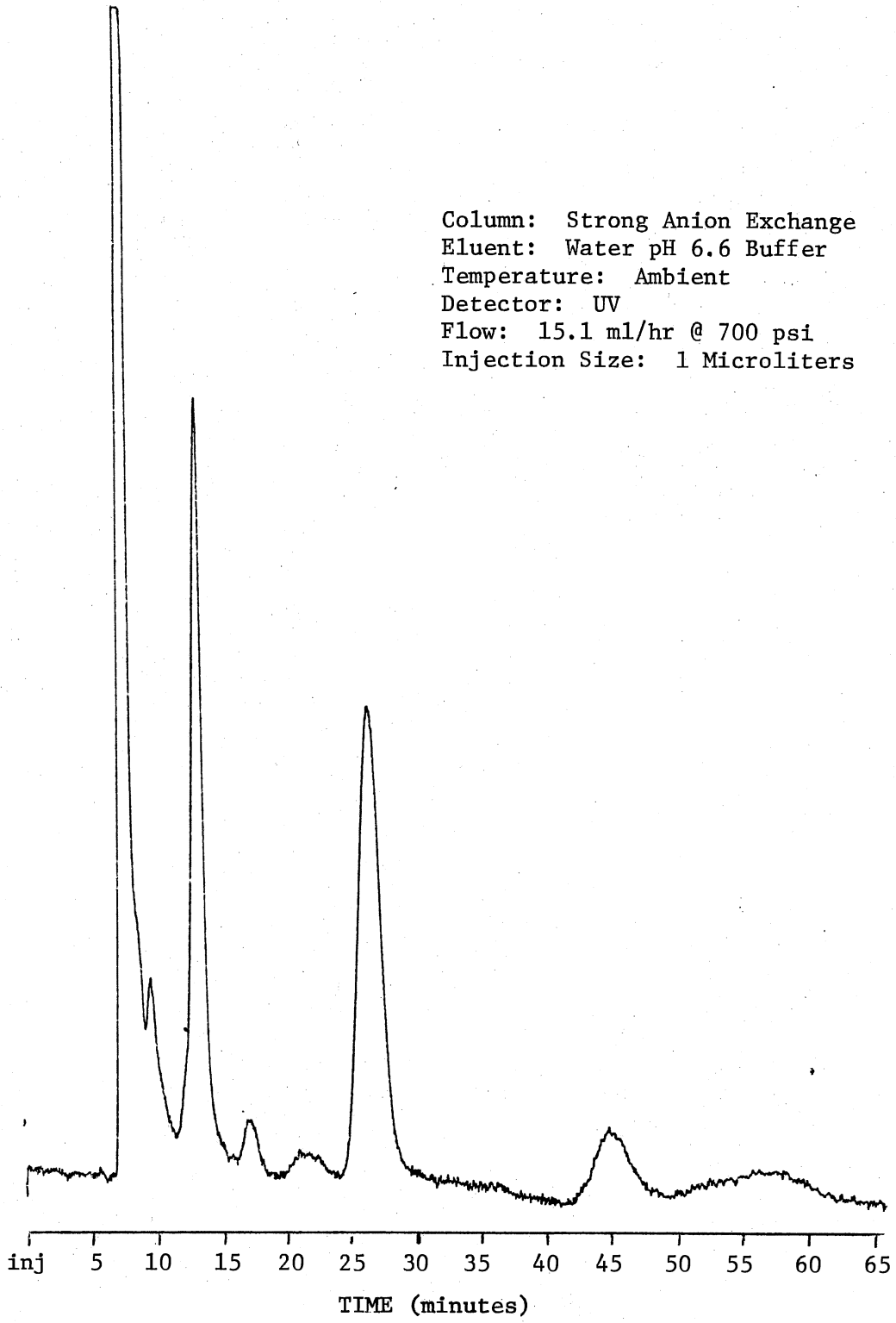

Figure 13. Chromatogram of Urine Components

instrument. Many of these modifications are described in Chapter IV while their effect can best be seen in the data obtained.

Separation of this standard mixture has proven the usefulness of this chromatographic system to complex mixtures. Analysis times were all within 45 minutes which is far quicker than many previous methods. The high selectivity of the detectors in this investigation has unavoidably reduced the number of compounds that can be detected.

The applications described in this chapter demonstrate the capabilities of this liquid chromatograph. The concentration methods are probably responsible for the limited number of peaks obtained. Nevertheless liquid chromatography has proven to be a useful tool in the analysis of organic nitrogen compounds.

Future Studies

The separations performed in this investigation have used the full capabilities of the existing chromatograph. Future studies of organics in water or trace analysis in general would be enhanced by several instrument modifications.

The first change would be the installation of a high pressure pump capable of delivering large volumes. This would make gradient elution possible and allow better and faster separation. A sampling valve would then be necessary at the higher pressures and would permit the use of larger injection sizes. These two modifications coupled with preparative columns and a detector sensitive to a wider range of compound types would increase the chances of mass spectral identification.

Future work is still needed in the preparation of specifically labeled isotopic standards. Synthesis of these standards can be obtained by either biological or chemical reactions. Separation of the mixtures into pure standards without inducing rearrangement of the molecules or isotopic label may be best achieved by high performance liquid chromatography using preparative columns.

SELECTED BIBLIOGRAPHY

(1) Allen, Herbert E. and J. R. Kramer, "Nutrients in Natural Waters," John Wiley and Sons, New York, New York, 1972.

(2) Domogalla, B. P., C. Juday and W. H. Peterson, J. Biol. Chem., 23, 269 (1925).

(3) White, G. C., "Handbook of Chlorination," Van Nostrand Reinhold Co., New York, New York, 1972.

(4) Ciaccio, L. L., ed., "Water and Water Pollution Handbook," Vol. IV Marcel Derkker, Inc., New York, New York, 1971.

(5) Erdman, Gordon J., M. M. Everett and W. E. Hanson, Science, 124, 1026 (1956).

(6) Feth, J. H., Water Resour. Res., 2, 41 (1966).

(7) Ciaccio, L. L., ed., "Water and Water Pollution Handbook," Vol. II Marcel Derkker, Inc., New York, New York, 1971.

(8) Peterson, W. H., E. F. Fred and B. P. Domogalla, J. Biol. Chem., 23, 287 (1925).

(9) Stewart, D. P. W., Nature, 200, 1020 (1963).

(10) Chau, Y. K. and J. P. Riley, Deep-Sea Research, 13, 1115 (1966).

(11) Katz, Sidney, W. W. Pitt and C. Scott, Water Research, 6, 1029 (1972).

(12) Park, Kilho, J. M. Prescott, W. T. Williams and D. W. Hood, Science, 128, 531 (1962).

(13) Tatsumoto, Mitsunobu, W. T. Williams, J. M. Prescott and D. W. Hood, J. Marine Research, 19, 89 (1961).

(14) Bark, L. S., R. L. Cooper and K. C. Wheatstone, Water Research, 6, 117 (1973).

(15) Caruso, S. C., H. C. Bramer and R. D. Hoak, Int. J. Air and Water Pollut., 10, 41 (1966).

(16) Litchfield, Carl D. and J. M. Prescott, Limnol Ocean, 15, 250, (1970).

(17) Mieure, J. P. and M. W. Dietrich, J. Chromatog., 11, 559 (1973).

(18) Degens, Egon T., J. H. Reuter and K. N. F. Shaw, Geochimica et Cosmochimica Acta., 28, 45 (1964).

(19) Palmork, Karsten H., Acta Chem. Scand., 17, 1456 (1963).

(20) Ryckman, D. W., J. W. Irvin and R. H. F. Young, J. Water. Pollut. Cont. Fed., 39, 458 (1965).

(21) Ryckman, D. W., N. C. Birbank, Jr. and E. Edgerley, Jr., J. Amer. Water Works Assoc., 56, 974 (1964).

(22) Jeffery, Lela M. and D. W. Hood, J. Marine Research, 17, 247 (1958).

(23) Junk, G. A., et al., Anal. Chem., 99, 745 (1974).

(24) Burnham, A. K., et al., Anal. Chem., 44, 139 (1972).

(25) Parsons, T. R. and J. D. H. Strickland, Science, 136, 313 (1962).

(26) Ertingshausen, Gerard, H. J. Adler and A. S. Reichler, J. Chromatog., 42, 355 (1969).

(27) Burnham, A. K., et al., J. Amer. Water Works Assoc., 65, 722 (1973).

(28) Hobbie, John E. and K. L. Webb, Science, 159, 1463 (1968).

(29) Streuli, Carl A., ed., "Analytical Chemistry of Nitrogen and Its Compounds," John Wiley and Sons, New York, New York, 1970.

(30) Riley, J. P. and D. Taylor, Analatica. Chimica. Acta., 46, 307, (1969).

(31) Ertingshausen, Gerard and H. J. Alder, J. Chromatog., 44, 620 (1967).

(32) Udenfriend, Sidney, et al., Science, 24, 871 (1972).

(33) Klein, Peter D. and P. A. Szczepanik, Anal. Chem., 39, 1276 (1967).

(34) Litchfield, Carol D. and D. W. Hood, Appl. Microbiol., 14, 145 (1966).

(35) Twsett, M., Proc. Warsaw Soc. Nat. Sci. Biol., Sec. 14, No. 6 (1903).

(36) Martin, J. P. and B. L. M. Synge, J. Biochem., 35, 1358 (1941).

(37) Kirkland, J. J., J. Chromatog. Sci., 7, 7 (1969).

(38) Thacker, L. H., W. W. Pitt, Jr., S. Katz and C. D. Scott, Clin. Chem., 16, 626 (1970).

(39) Snyder, L. R., J. Chromatog. Sci., 7, 352 (1969).

(40) Kirkland, J. J., "Modern Practices of Liquid Chromatography," Wiley-Interscience, New York, New York, 1971.

(41) Bailey, F. and P. N. Brittian, J. Chromatog., 83, 431 (1973).

(42) Knox, J. H. and J. Jurand, J. Chromatog., 87, 95 (1973).

(43) Konecny, J., J. Felber and J. Gruner, J. Antibiotics, 26, 135 (1973).

(44) Sleight, R. B., J. Chromatog., 83, 31 (1973).

(45) Lindstrom, J. Chromatog., 100, 189 (1974).

APPENDIX

TABLE IV

FLUORESCENCE CHARACTERISTICS OF SELECTED COMPOUNDS

Compound	Activation Maximum (nm)	Fluorescence Maximum (nm)
Aniline	280	340
Uracil	270	315
Uric Acid	325	370
Indole	280	350
Tyrosine	275	310
Tryptophan	285	365
Phenylalanine	360	282
Adenine	272	380

VITA

Jerry Dale Caplinger

Candidate for the Degree of

Master of Science

Thesis: THE DETERMINATION OF ORGANIC NITROGEN COMPOUNDS IN WATER BY HIGH SPEED LIQUID CHROMATOGRAPHY

Major Field: Chemistry

Biographical:

Personal Data: Born in Oklahoma City, Oklahoma, January 22, 1949, the son of Mr. and Mrs. Bill Caplinger.

Education: Graduated from Piedmont High School, Piedmont, Oklahoma, in May, 1967; received the Bachelor of Science degree from Central State University, Edmond, Oklahoma in May, 1972 with majors in Mathematics and Chemistry; completed requirements for the Master of Science degree at Oklahoma State University in May, 1976.

Professional Experience: Graduate teaching assistant at Oklahoma State University during 1972, 1973, 1974, 1975, and 1976; graduate research assistant at Oklahoma State University, September, 1973 to May, 1974 and September, 1975 to May, 1976 (sponsored by the Environmental Protection Agency), and September, 1973 to June 1974, and September, 1974 to December, 1974 (sponsored by the Atomic Energy Commission); recipient of Continental Oil Fellowship during the summers of 1973 and 1974, and Sun Oil Fellowship during the summer of 1975.

Professional Organizations: Officer of Phi Lambda Upsilon.