

ABSTRACT

SALLEY, CINDY T. An Improved Analytical Method for the Analysis of Reactive Dyes by High Performance Liquid Chromatography. (Under the direction of Keith R. Beck).

High Performance Liquid Chromatography or HPLC is a technique that is commonly used for the analysis of many different compounds. For the analysis of reactive dyes, ion-pair chromatography (IPC) is often implemented, but previous methods in the literature are a decade old and have not allowed for the analysis of highly complex dyes such as Reactive Blue 21, a copper phthalocyanine reactive dye.

In this work, IPC of reactive dyes is improved by examining a variety of different factors. Factors examined were ion-pair reagents and column stationary phases. Gradient adjustments, including gradient elution, curve, and time were examined to improve the analysis of Reactive Blue 21. Reactive Blue 21 is further examined through hydrolysis and two dyeings, exhaust dyeing and pad batch. A proposed grouping of peaks, referring to the structure of Reactive Blue 21, is given from the hydrolysis experiment, and the peaks are comparable to those shown in the two dyeings. Tetrabutylammonium bromide (TBAB), an ion pair reagent, along with the Atlantis C18 column provided the best analysis for the reactive dyes studied. For Reactive Blue 21, decreasing the slope of the gradient curve greatly increased the number of peaks resolved. Subsequently, these peaks could be ascribed to the hydroxyethylsulfone (HES), vinyl sulfone (VS), or sulfatoethylsulfone (SES) forms of the dye based on the rate of change of peak percent area shown in the hydrolysis and dyeing experiments.

**AN IMPROVED ANALYTICAL METHOD FOR THE ANALYSIS OF
REACTIVE DYES BY HIGH PERFORMANCE LIQUID
CHROMATOGRAPHY**

by

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DEDICATION

This thesis is dedicated to the memory of my grand-aunt, Ms. Christine Durham, who was a person that I always admired and was one of the most influential people in my upbringing. Her charm, wit, and determination made her a constant joy to be around and she always pushed me to pursue my dreams and to always respect and gain wisdom from my elders.

BIOGRAPHY

Cindy Salley was born in Portsmouth, VA on November 9, 1982 as the first and only child of Sidney and Merriel Salley. In 1999, Cindy received an Advanced Studies Diploma with Magnet Distinction from Israel Charles Norcom High School in Portsmouth, VA. She attended Spelman College in Atlanta, GA where she received a Bachelor of Science Degree in Chemistry in May of 2003. Her experiences conducting various scientific research as well as an encounter with a conservation scientist led Cindy to North Carolina State University, and more specifically, the College of Textiles. She is a candidate for the Master of Science in Textile Chemistry. On December 18, 2004, she married Elmo Sampson, III of Portsmouth, VA. Following completion of the Master of Science degree, Cindy will relocate to Johnstown, PA and pursue interests in the field of Chemistry.

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TABLE OF CONTENTS

	Page
LIST OF TABLES	viii
LIST OF FIGURES	x
1. INTRODUCTION	1
2. LITERATURE REVIEW	2
2.1 High Performance Liquid Chromatography	2
2.1.1 Overview of Chromatography	2
2.1.2 HPLC System	3
2.1.2.1 Detectors	5
2.1.2.2 Columns	5
2.2 Ion Pair Chromatography	6
2.2.1 Ion-Pair Reagents	7
2.2.2 Advantages of IPC	9
2.2.3 Disadvantages of IPC	9
2.3 Reactive Dyes	10
2.4 Analysis of Reactive Dyes	14
2.4.1 UV-Visible Spectroscopy	14
2.4.2 Thin Layer Chromatography	14
2.4.3 Capillary Electrophoresis	16
2.4.4 High Performance Liquid Chromatography	16
2.4.4.1 Online HPLC	17
2.4.4.2 LC-MS	19
3. EXPERIMENTAL METHODS AND PROCEDURES	21
3.1 Materials	21

3.1.1	Mobile Phase Components	21
3.1.2	Mobile Phase Preparation	22
3.1.3	Dyes	22
3.1.4	Chromatographic Columns	26
3.1.5	Chemicals for Experiments	28
3.2	Instrumentation	28
3.2.1	UV-Visible Spectroscopy	28
3.2.2	High Performance Liquid Chromatography System	28
3.2.2.1	Breeze HPLC System	29
3.2.2.2	Alliance HPLC System	29
3.3	Experiments	30
3.3.1	Ion-Pair Reagents	30
3.3.2	Stationary Phases	31
3.3.2.1	Stationary Phase	31
3.3.2.2	Gradient Adjustments	32
3.4	C.I. Reactive Blue 21 Hydrolysis	33
3.4.1	Hydrolysis	33
3.4.2	Exhaust Dyeing	33
3.4.3	Pad Batch Dyeing	34
4.	RESULTS AND DISCUSSIONS	35
4.1	Ion-Pair Experiments	35
4.1.1	TBAB and TBAHS on Symmetry Column	35
4.1.2	TBAB and TBAHS on Novapak Column	41
4.1.3	C. I. Reactive Blue 21 on Atlantis Column	46
4.2	Stationary Phase and Gradient Experiments	49
4.2.1	Stationary Phases	49
4.2.2	C.I. Reactive Blue 21 Gradient Adjustments	53
4.2.2.1	Gradient Elution	53
4.2.2.2	Gradient Curve	57

4.2.2.3 Gradient Time	62
4.2.2.4 Gradient Optimization	71
4.3 Reactive Blue 21 Experiments	74
4.3.1 Hydrolysis of Reactive Blue 21	75
4.3.2 Exhaust Dyeing with Reactive Blue 21	79
4.3.3 Pad Batch Dyeing of Reactive Blue 21	82
5. CONCLUSIONS	84
6. RECOMMENDATIONS FOR FUTURE WORK	86
7. REFERENCES	87

LIST OF TABLES

		Page
Table 3.1	Commercial and C.I. Names of Dyes	23
Table 3.2	Columns for HPLC Analysis	27
Table 3.3	Guard Cartridges	27
Table 3.4	Gradient 1	30
Table 3.5	Columns and Dyes Examined by Ion-Pair Reagents	31
Table 3.6	Gradient 2	32
Table 3.7	Optimized Gradient for C.I. Reactive Blue 21	32
Table 4.1	Number of Resolved Peaks on Symmetry (C18) Column and Gradient 1	40
Table 4.2	k' Values on Symmetry (C18) Column and Gradient 1	40
Table 4.3	Number of Resolved Peaks on Novapak (C18) Column and Gradient 1	46
Table 4.4	k' Values on Novapak Column	46
Table 4.5	Resolved Peaks and k' Values for C.I. Reactive Blue 21 on Atlantis Column	49
Table 4.6	Gradients 1 and 2	50
Table 4.7	Column Comparison for Reactive Dyes	50
Table 4.8	C. I. Reactive Blue 21 Atlantis (C18) Gradient Elution Adjustment Example	54
Table 4.9	Number of Peaks Resolved for C.I. Reactive Blue 21 Gradient Changes	57

Table 4.10	Number of Peaks Resolved for C.I. Reactive Blue 21 Gradient Curve	62
Table 4.11	Gradient Time Adjustment Example	63
Table 4.12	Number of Resolved Peaks for C.I. Reactive Blue 21 Time	71
Table 4.13	Number of Resolved Peaks for C.I. Reactive Blue 21 Optimization	72
Table 4.14	Peaks of Interest for C.I. Reactive Blue 21 Hydrolysis	78
Table 4.15	Peaks of Interest for C.I. Reactive Blue 21 Exhaust Dyeing	82

LIST OF FIGURES

	Page
Figure 2.1 Schematic Diagram of an HPLC System	4
Figure 2.2 Possible Mechanism Sketch of IPC	7
Figure 2.3 Ion-Pair Formation	8
Figure 2.4 Triazines and Vinyl Sulfone Groups of Reactive Dyes	12
Figure 2.5 Diagram of Hydrolysis Reaction by Mock	13
Figure 3.1 Ion-Pair Reagents	22
Figure 3.2 Structure of C. I. Reactive Red 43	23
Figure 3.3 Structure of C. I. Reactive Red 180	24
Figure 3.4 Structure of C. I. Reactive Red 239	24
Figure 3.5 Structure of C. I. Reactive Yellow 3	24
Figure 3.6 Structure of C. I. Reactive Yellow 135	25
Figure 3.7 Structure of C. I. Reactive Blue 238	25
Figure 3.8 Structure of C. I. Reactive Blue 21	26
Figure 3.9 Ion-Pair Reagent Mobile Phase	30
Figure 4.1 C. I. Reactive Red 239 on Symmetry (C18) Using TBAB and Gradient 1	36
Figure 4.2 C. I. Reactive Red 239 on Symmetry (C18) Using TBAHS and Gradient 1	36
Figure 4.3 C. I. Reactive Yellow 3 on Symmetry (C18) Using TBAB and Gradient 1	37
Figure 4.4 C. I. Reactive Yellow 3 on Symmetry (C18) Using TBAHS and Gradient 1	37

Figure 4.5	C. I. Reactive Yellow 135 on Symmetry (C18) Using TBAB and Gradient 1	38
Figure 4.6	C. I. Reactive Yellow 135 on Symmetry (C18) Using TBAHS and Gradient 1	38
Figure 4.7	C. I. Reactive Yellow 168 on Symmetry (C18) Using TBAB and Gradient 1	39
Figure 4.8	C. I. Reactive Yellow 168 on Symmetry (C18) Using TBAHS and Gradient 1	39
Figure 4.9	C. I. Reactive Red 180 on Novapak (C18) Using TBAB and Gradient 1	41
Figure 4.10	C. I. Reactive Red 180 on Novapak (C18) Using TBAHS and Gradient 1	42
Figure 4.11	C. I. Reactive Red 239 on Novapak (C18) Using TBAB and Gradient 1	42
Figure 4.12	C. I. Reactive Red 239 on Novapak (C18) Using TBAHS and Gradient 1	43
Figure 4.13	C. I. Reactive Yellow 3 on Novapak (C18) Using TBAB and Gradient 1	43
Figure 4.14	C. I. Reactive Yellow 3 on Novapak (C18) Using TBAHS and Gradient 1	44
Figure 4.15	C. I. Reactive Yellow 168 on Novapak (C18) Using TBAB and Gradient 1	44
Figure 4.16	C. I. Reactive Yellow 168 on Novapak (C18) Using TBAHS and Gradient 1	45
Figure 4.17	C. I. Reactive Blue 21 on Novapak (C18) Using TBAB and Gradient 1	47
Figure 4.18	C. I. Reactive Blue 21 on Symmetry (C18) Using TBAHS and Gradient 1	47

Figure 4.19	C. I. Reactive Blue 21 on Atlantis (C18) Using TBAB and Gradient 1	48
Figure 4.20	C. I. Reactive Blue 21 on Atlantis (C18) Using TBAHS and Gradient 1	48
Figure 4.21	C. I. Reactive Blue 21 on Novapak (C18) (3-Good) and Gradient 1	51
Figure 4.22	C. I. Reactive Blue 21 on Zorbax Reliance (C18) (2-Better) and Gradient 1	51
Figure 4.23	C. I. Reactive Blue 21 on Atlantis (C18) (1-Best) and Gradient 1 ...	52
Figure 4.24	C. I. Reactive Blue 21 Atlantis (C18) with 90_10 Gradient	54
Figure 4.25	C. I. Reactive Blue 21 Atlantis (C18) with 80_20 Gradient	55
Figure 4.26	C. I. Reactive Blue 21 Atlantis (C18) with 70_30 Gradient	55
Figure 4.27	C. I. Reactive Blue 21 Atlantis (C18) with 65_35 Gradient	56
Figure 4.28	C. I. Reactive Blue 21 Atlantis (C18) with 60_40 Gradient	56
Figure 4.29	Profile of Gradient Curves	58
Figure 4.30	C. I. Reactive Blue 21 Atlantis (C18) with Curve 2 and Gradient 1	59
Figure 4.31	C. I. Reactive Blue 21 Atlantis (C18) with Curve 4 and Gradient 1	59
Figure 4.32	C. I. Reactive Blue 21 Atlantis (C18) with Curve 6 and Gradient 1	60
Figure 4.33	C. I. Reactive Blue 21 Atlantis (C18) with Curve 7 and Gradient 1	60
Figure 4.34	C. I. Reactive Blue 21 Atlantis (C18) with Curve 8 and Gradient 1	61
Figure 4.35	C. I. Reactive Blue 21 Atlantis (C18) with Curve 9 and Gradient 1	61
Figure 4.36	C. I. Reactive Blue 21 Atlantis (C18) with Curve 10 and Gradient 1	62
Figure 4.37	C. I. Reactive Blue 21 Atlantis (C18) with 1.5_3.5 Gradient Time	64
Figure 4.38	C. I. Reactive Blue 21 Atlantis (C18) with 2_4 Gradient Time	64

Figure 4.39	C. I. Reactive Blue 21 Atlantis (C18) with 3_5 Gradient Time	65
Figure 4.40	C. I. Reactive Blue 21 Atlantis (C18) with 4_6 Gradient Time	65
Figure 4.41	C. I. Reactive Blue 21 Atlantis (C18) with 5_7 Gradient Time	66
Figure 4.42	C. I. Reactive Blue 21 Atlantis (C18) with 6_8 Gradient Time	66
Figure 4.43	C. I. Reactive Blue 21 Atlantis (C18) with 7_9 Gradient Time	67
Figure 4.44	C. I. Reactive Blue 21 Atlantis (C18) with 8_10 Gradient Time	67
Figure 4.45	C. I. Reactive Blue 21 Atlantis (C18) with 9_11 Gradient Time	68
Figure 4.46	C. I. Reactive Blue 21 Atlantis (C18) with 10_12 Gradient Time ...	68
Figure 4.47	C. I. Reactive Blue 21 Atlantis (C18) with 11_13 Gradient Time ...	69
Figure 4.48	C. I. Reactive Blue 21 Atlantis (C18) with 12_14 Gradient Time ...	69
Figure 4.49	C. I. Reactive Blue 21 Atlantis (C18) with 13_15 Gradient Time ...	70
Figure 4.50	C. I. Reactive Blue 21 Atlantis (C18) with 14_16 Gradient Time ...	70
Figure 4.51	C. I. Reactive Blue 21 Atlantis (C18) with 10_12 Gradient Time, 70/30 Elution and C7	72
Figure 4.52	C. I. Reactive Blue 21 Atlantis (C18) with 10_12 Gradient Time, 60/40 Elution and C6	73
Figure 4.53	C. I. Reactive Blue 21 Atlantis (C18) with 10_12 Gradient Time, 60/40 Elution and C7	73
Figure 4.54	C. I. Reactive Blue 21 Prior to Adding Base for Hydrolysis	75
Figure 4.55	C. I. Reactive Blue 21 Hydrolysis at 65°-10 Minutes After Base was Added	76
Figure 4.56	C. I. Reactive Blue 21 Hydrolysis at 65°-25 Minutes After Base was Added	76
Figure 4.57	C. I. Reactive Blue 21 Hydrolysis at 65°-50 Minutes After Base was Added	77

Figure 4.58	Hydrolysis of C. I. Reactive Blue 21 Predicted Dye Forms	79
Figure 4.59	C. I. Reactive Blue 21 Prior to Adding Base for Exhaust Dyeing ...	80
Figure 4.60	C. I. Reactive Blue 21 Exhaust Dyeing After Na ₂ CO ₃ Addition	80
Figure 4.61	C. I. Reactive Blue 21 Exhaust Dyeing-40 Minutes After Na ₂ CO ₃ Addition	81
Figure 4.62	C. I. Reactive Blue 21 Prior to Adding Base for Pad Batch Dyeing	83
Figure 4.63	C. I. Reactive Blue 21 Pad Batch Dyebath (next day)	83

1. INTRODUCTION

The primary focus of the present work is to develop a more efficient way of analyzing reactive dyes using High Performance Liquid Chromatography (HPLC), a widely used technique in the scientific community. The literature review that follows will cover information dealing with HPLC, ion-pair chromatography (IPC), reactive dyes, and other analytical methods for studying reactive dyes. IPC is often used for the analysis of reactive dyes to examine characteristics of the dye, such as the rate of hydrolysis. However, earlier methods are not as useful for analyzing some highly complex reactive dyes. The development of an improved procedure for analysis is needed to reflect advances in technology and to examine more complex reactive dyes.

Two different ion-pair reagents will be examined as well as columns with varying column characteristics (length, inner diameter, and particle size). Gradient adjustments will be examined to optimize the analysis of Reactive Blue 21, and an improved method for analysis of this dye was developed. Hydrolysis, as well as exhaust and pad batch dyeing, were also conducted on Reactive Blue 21.

2. LITERATURE REVIEW

2.1 High Performance Liquid Chromatography (HPLC)

2.1.1 Overview of Chromatography

Column chromatography was first developed as a tool for separation science by Tswett, who used fine particles of calcium carbonate to pack a glass column in order to separate a mixture of plant pigments. This led to discoveries by scientists such as Martin and Synge, who are credited with discovering modern liquid chromatography, from which HPLC was born [1, 2]. The two major components that allow for separation of components in a sample are the liquid mobile phase and a stationary phase, which can be solid or liquid (bonded on a solid support) [1-4].

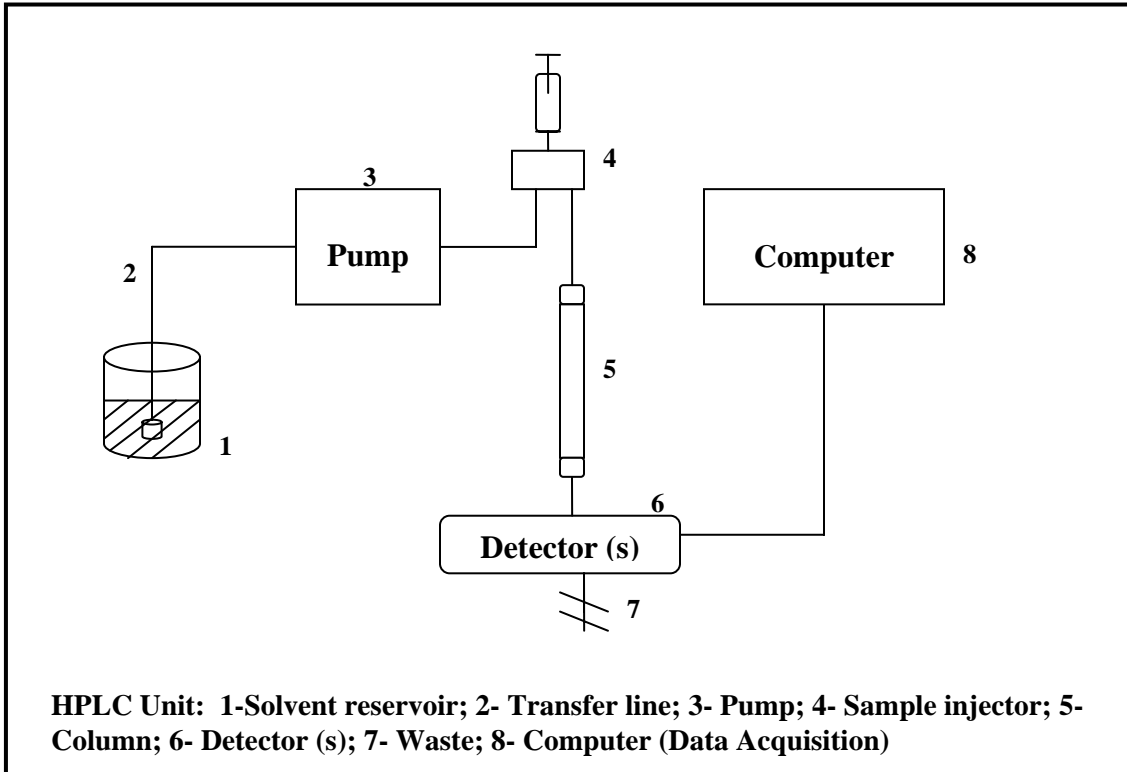
Chromatography is classified according to the mechanism used to interact with the solute (sample), and the five major types of chromatography are adsorption, ion-exchange, molecular exclusion, affinity, and partition. Adsorption chromatography utilizes a solid stationary phase that adsorbs solutes being passed through a column using a liquid mobile phase. The stronger the adsorption of the solute, the longer it takes to pass through a column and be analyzed. This is also known as normal phase chromatography, and includes thin-layer chromatography (TLC), though a column is not used for this method. Ion-exchange chromatography (IEC) is often used to analyze samples that contain ionic groups, such as amino acids. The stationary phase in IEC consist of anions (i.e. $-\text{SO}_3^-$, $-\text{COO}^-$) or cations (i.e. $-\text{NH}_3^+$, $-\text{N}(\text{CH}_3)_3^+$) that are attached to a solid phase, which is typically a resin. Samples

are introduced through a liquid mobile phase, and sample ions of the opposite charge of the resin are attracted to the stationary phase. A special type of IEC is ion chromatography, which is used to separate organic acids and inorganic ions. Molecular exclusion chromatography, also known as gel permeation chromatography, or gel filtration chromatography, is separation based on molecular size and can analyze compounds like polymers. The mobile phase carries the sample through the stationary phase, which is actually a porous gel. Larger molecules elute first, since they are too big to enter the pores, while the smaller molecules enter the gel, and take longer to move through the column. Affinity chromatography is a very selective analytical procedure used on compounds like proteins and lipids. Separation is achieved when the stationary phase only retains certain groups of molecules, like a specific type of antigen. Partition chromatography involves a liquid stationary phase, which is bonded to a solid surface like silica, and a liquid or gaseous mobile phase. The solute partitions into the stationary phase, which allows for analyte separation, and this is the type of chromatography from which HPLC was born [1, 4].

2.1.2 HPLC System

High performance liquid chromatography is one of the most popular techniques used by scientists to separate mixtures into components. HPLC utilizes high pressure to force a solvent (mobile phase) through a column. The basic components of an HPLC System include: mobile phase reservoirs, pump, sample injector, column, and detector. A basic diagram of HPLC can be seen in Figure 2.1:

Figure 2.1: Schematic Diagram of an HPLC System



Solvent reservoirs are utilized to store the mobile phase, which should be filtered and degassed before it is used. The solvent is then transferred from these reservoirs into a transfer line by a pump. Depending on the HPLC system and if utilizing a gradient method, the solvents could be mixed through a solvent proportioning valve before going through the pump, or could be mixed after the solvent flows through the pump. As the solvent or mobile phase is running through the system, a sample can be introduced by an injector onto the column. The sample migrates through the column, and is analyzed by a detector on the other end of the column. The information collected from the detector is sent to a computer or data readout, from which a chromatogram is obtained, while the solvent utilized for the

experiment and samples are sent to waste [1, 3, 4].

2.1.2.1 Detectors

An HPLC system is able to utilize different detectors, and this allows for the system to be able to analyze a wide variety of samples. The most popular detector for HPLC is the UV detector. Provided that a compound of interest can absorb light in the UV-Visible region, a UV detector is ideal since it is fairly sensitive, fit for gradient elutions, and has a wide linear range [4]. A basic UV detector allows for one or two wavelengths to be recorded for analysis, but others have monochromators in them, which allow for the full wavelength range of the spectra to be recorded. These types of UV-Visible detectors are called photodiode array (PDA) detectors, and can be useful for sample components that absorb light at different wavelengths. For more sensitive analytes, a fluorescence detector could be used, but if a compound is not fluorescent, a fluorescent group must be tagged to the compound for it to be recognized by the detector. A refractive index detector is very universal, because it can respond to just about any analyte, but has a lower detection limit (1000x less than UV) and is not good for gradient elution [1]. For more complex mixtures, other analytical techniques can be coupled to HPLC, such as mass spectrometry [5-8] or FTIR [9].

2.1.2.2 Columns

The column is a key component in the HPLC system, since it is the basis for the separation mechanism. Columns are made of smooth-bore stainless steel and typically have liquid stationary phases bonded to a solid, which is often silica. The silica is modified at the silanol group in order for functional groups, which interact with the sample to achieve separation. Some of the functional groups added include butyl (C₄), phenyl (C₆H₅),

octadecyl (C₁₈), amino (NH₂), etc. Columns originally ranged from 10 to 30cm in length, 4 to 10mm in inner diameter (i.d.), and particle size packings from 5 to 10µm [3]. However, newer technologies have allowed for columns to be packed with particles as small as 1.7µm, to have i.d.'s as narrow as 1.0-2.1mm, and lengths shorter than 50mm. In addition, application-specific columns that can analyze specific types of samples such as chiral molecules, environmental samples, and peptide molecules are available [10]. This is achieved by adjusting column factors such as chemistry of the stationary phase by adding ligand groups, embedded polar groups, or difunctional bondings in silica [11]. With the development of such columns, improved separation can be achieved better than before for analytes targeted for that specific column [10].

2.2 Ion Pair Chromatography (IPC)

Ion pair chromatography is a form of reverse-phase high performance liquid chromatography (RP-HPLC) that is utilized for the chromatographic separation and determination of ionic species. IPC is a sister alternative to ion-exchange chromatography, which uses columns packed with anion-exchange or cation-exchange resins for analysis of ionic species. Ion exchange chromatography is best used for samples with small inorganic and organic ions, such as those found in nucleotides. However, ion-exchange chromatography is not ideal when dealing with mixtures of bases, acids, and certain neutral products, and IPC is capable of dealing with all of these. Reversed phase chromatography, allows for separation of charged species that would not otherwise be suitable for analysis. The mobile phase in ion-pair chromatography consists of an aqueous buffer with an organic

solvent (preferably methanol or acetonitrile) and the ion pair reagent [3-4].

2.2.1 Ion-Pair Reagents

An ion-pair reagent contains an ion that interacts with a sample component of opposite charge. Figure 2.2 below is a sketch of how a sample (dye) interacts with the IPA on a reversed phase column in HPLC. Here, the TBAB behaves like a small surfactant, where the hydrophobic chain interacts with the dye increasing its retention. Ion-pair formation can be achieved for a cationic sample that interacts with a negative ion, and an anionic sample that interacts with a positive ion, which is simplified in Figure 2.3.

Figure 2.2: Possible Mechanism Sketch of IPC

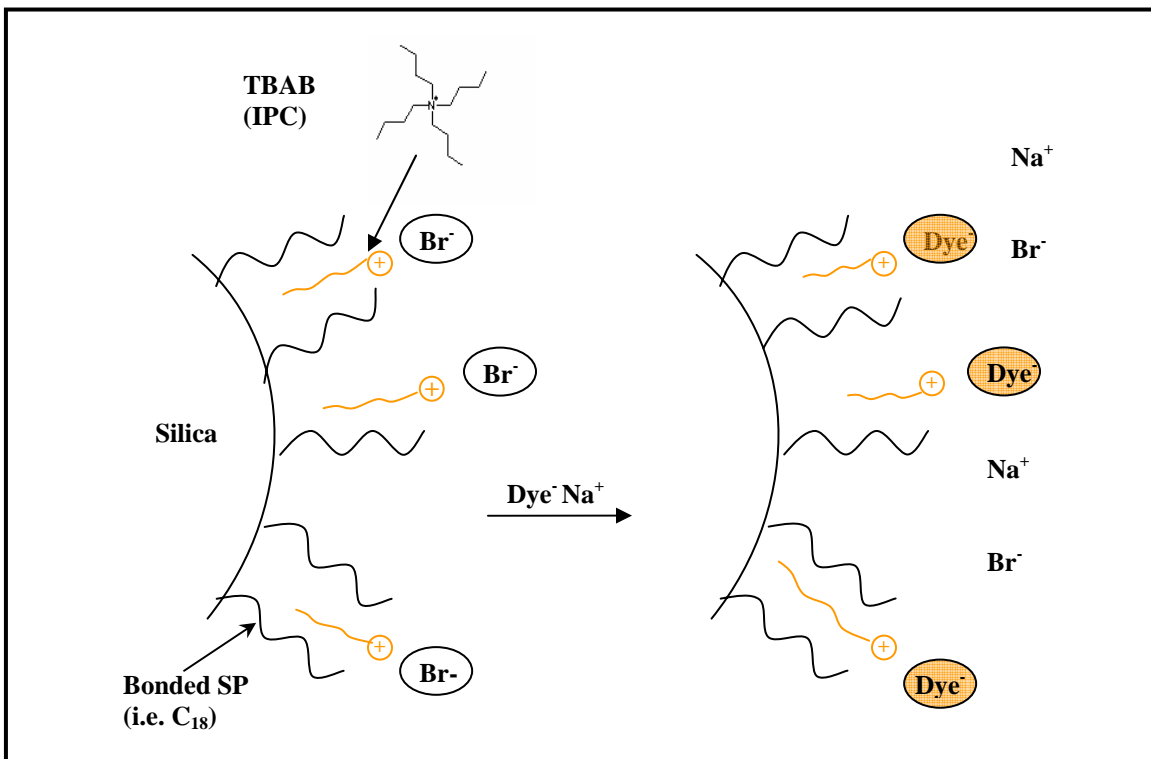
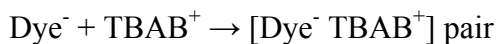
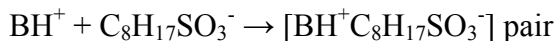


Figure 2.3: Ion-Pair Formation



In IPC, it is essential to select an ion-pair reagent that is most suitable for the analytes of interest. When choosing an ion-pair reagent (IPA), consideration should be given to pKa values of analytes and the type of mixture being analyzed. For example, for the analysis of sulfonated dyes, quaternary amines such as tetrabutylammonium phosphate are recommended for IPC analysis [3-4].

To analyze structures that contain a variety of charged compounds, such as amino acids or sugars, more than one ion pair reagent can be used for analysis. Meyer uses a variety of ion-pair reagents to analyze three different types of natural organic compounds, which include uronic acids, amino sugars, and carbohydrates. These compounds were being analyzed after being derivatized by *p*-aminobenzoic acid for fluorescent tagging. The ion pairing agents used in this experiment include hexadecyltrimethylammonium bromide, tetrabutylammonium hydrogen phosphate, tetraethyl ammonium bromide, tetramethylammonium bromide, and tetrapentylammonium iodide. Combinations of these were examined along with columns, and though some mixtures influenced retention, a single ion pairing agent, tetrabutylammonium hydrogen phosphate, yielded the best separation [12].

For reactive dye analysis, Zotou used 1mM cetyltrimethylammonium bromide (CTAB) in the mobile phase for the analysis of the hydrolysis of flurotriazinic reactive dyes, Cibacron Yellow F-4G and Cibacron Blue F-R [13]. Kanazawa also employed CTAB in the

mobile phase to examine a monoazo dye [14]. However, the most common ion pairing agent utilized for reactive dyes is tetrabutylammonium bromide (TBAB). This IPA is one of the first used in the literature for dye analysis [15-16], and is a primary choice particularly for the study of reactive dye hydrolysis [15-19]. TBAB has also been used not only as an IPA in chromatography, but it is also used as a reagent for selective dye extraction [20].

2.2.2 Advantages of IPC

Ion-pair chromatography is utilized in reverse phase systems, so to employ the ion-pair mechanism, a mobile phase change is made. Not only can different acid mixtures or base mixtures be analyzed with this technique, but also amphoteric molecules, which include one cationic and one anionic group. This can not be accomplished through ion-exchange chromatography or reversed phase HPLC alone [4]. IPC can be a preferred technique of choice when the analytes have similar pKa values, since analyte selectivity can be influenced by the ion-pair reagent. Also, there are many ion-pair reagents, such as sodium octyl sulfate, tetrapropylammonium bromide, and perchloric acid, that are readily available for commercial use [4, 6].

2.2.3 Disadvantages of IPC

IPC has many obvious advantages, but it has some disadvantages. Though ion-pair reagents are readily available, depending on the technique being used, certain reagents can not be used successfully. This is particularly an issue when dealing with HPLC-MS, where MS detection can suffer with the presence of certain ion pairing reagents. Holčápek

examined this by observing the signal suppression of different IPAs in electrospray MS (for LC-MS) when sulphonated dyes were analyzed. So reagent selectivity is especially key in such cases [6-7]. Analysis can not be done over a wide pH range, and thus ion chromatography would be preferred in such cases. In order to maintain the appropriate levels of pH needed for experiments, a buffer is used in the mobile phase. This is not a huge problem, but in general, ion pair systems take time to equilibrate, so this does cause the preparation and closing steps for HPLC analysis to be lengthened by adding a water wash step at the beginning and end of each day of IPC runs or if the mobile phase composition needs to be changed [13]. In addition to this, though adjusting the mobile phase is all that is needed for IPC, it is recommended that a column that is being used for ion-pair chromatography should be distinguished for only IPC, since the ion-pair reagents tend to be harmful to silica [4].

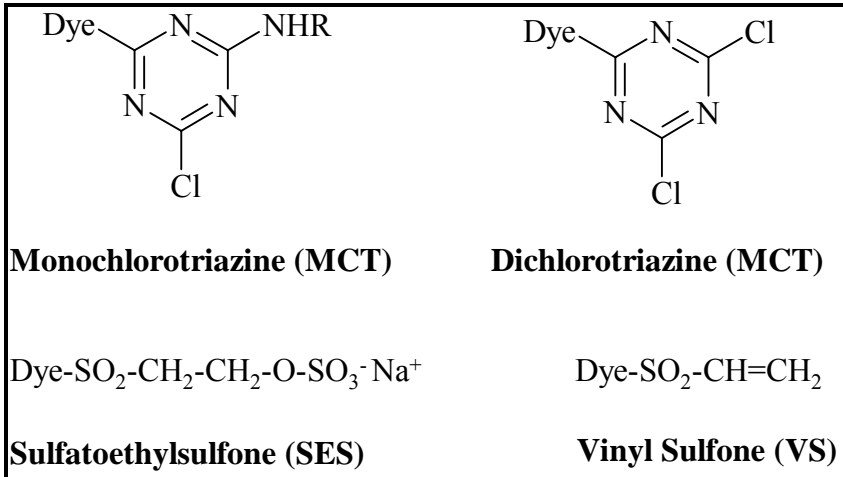
2.3 REACTIVE DYES

The origin of the earliest dye is very difficult to determine, but it is speculated that the first color applied to a textile was probably a juice or berry stained to a fabric like animal skin. Since then, different natural dyes have been utilized by many ancient civilizations, such as the Egyptians, Greeks, Indians, and Chinese to name a few [21]. However, the field of synthetic dyes is fairly new. In 1855, the first synthetic dye was a black vat dye named fuschin, which was made by Natanson, a professor at Warsaw University. A year later, William Henry Perkin synthesized mauve or aniline purple [21-22]. Today, there are over 8,000 different synthetic dyes with a wide range of structures and colors [22].

In 1956, ICI introduced a new class of dyes known as reactive dyes or fiber reactive dyes. Reactive dyes are different from other dye classes in that they form covalent bonds with fibers such as cotton, and they are currently one of the most popular types of dyes used today, because of their excellent properties [21-23]. Fiber reactive dyes typically have brilliant shades and the ability to be applied to a fabric using a variety of different applications. They are more stable to light and wet treatment than direct dyes, and have a more simplistic application than vat dyes [22]. However these dyes after covalently bonding with the fiber usually leaves a substantial amount of dye unfixed (from 10 to 40 percent). This unfixed dye is hydrolyzed during the dyeing process, and must be washed off, but is essentially wasted dye [21]. The wasted dye, especially those dyes that contain metal complexes, can pose an environmental problem since special waste treatment are required to comply with environmental regulations [24-25].

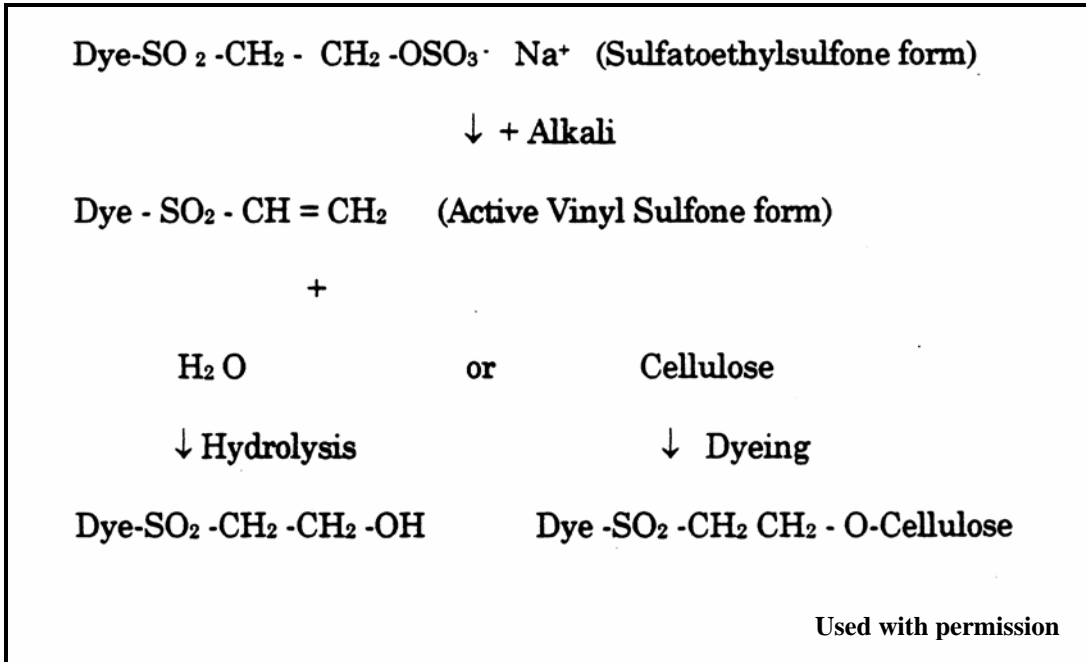
Reactive dyes are classified by the groups that facilitate covalent bonding to the fiber. Two of these reactive dye classifications are triazines (monochloro and dichloro) and vinylsulfones, which are shown below in Figure 2.4. The sulfatoethylsulfone is the reactive precursor to the dye, and in most vinylsulfone dyes, both forms are present [21-22].

Figure 2.4: Triazine and Vinyl Sulfone Groups of Reactive Dyes



Hydrolysis of a reactive dye occurs when alkali is introduced to an aqueous dye mixture. This mixture is typically heated to a certain temperature, and can be conducted with or without fabric. The general reaction for what occurs for a vinyl sulfone dye can be seen below in Figure 2.6 from Mock [21]. In the absence of a fabric, the dye is able to undergo hydrolysis only. In the presence of a fabric, hydrolysis takes place along with the dyeing process, and while hydrolysis does occur, the dye can attach to the cellulose (fabric) as well.

Figure 2.5: Diagram of Hydrolysis Reaction by Mock [21]



Fiber reactive dyes are usually applied by either pad-batch or exhaust dyeing. Exhaust dyeing occurs by step-by-step addition of dye and chemicals or they can all be added at the same time. When added at the same time, the dyebath starts at a low temperature and is gradually raised to a higher temperature. When added step-by-step, the dye is exhausted using salt, and the pH is allowed to change gradually so the dye can react with the fabric. In pad-batch dyeing, the mixture of dye and dyeing auxiliaries are padded on a flat woven fabric and allowed to stand while fixation takes place. After the reaction has occurred, the fabric is rinsed in cold water to remove the hydrolyzed dye. Hydrolysis is not desirable, because this decreases the amount of dye that will covalently bond to the fabric. [21, 26].

2.4 ANALYSIS OF DYES

There are a variety of different methods that can be used to analyze a dye including UV-Visible Spectroscopy (2.4.1), Thin Layer Chromatography (2.4.2), Capillary Electrophoresis (CE) (2.4.3), and the main focus of this study, HPLC (2.4.4).

2.4.1 UV-Visible Spectroscopy

An UV-Visible spectrophotometer is often used before HPLC work in order to determine λ_{\max} of organic compounds like dyes, but it can also be helpful for other analytical techniques [14]. Comparison of the UV-Visible spectra of natural dyes and dye extracts is a good way of determining how close the chemical identity of the dyed fabric extract is to the natural dye. For example, marigold had the same λ_{\max} for the dyed fabric extract and the natural dye as well as similar UV-Visible spectra, however the jackwood dye and dyed fabric extract had different UV-Visible spectra. This is important since the dye is not overly affected by the extraction method used for the dyed fabric in certain cases, but in others, a different method of dye extraction would be needed [27]. Bhattacharyya also used UV-Visible Spectrophotometry to determine if any interactions had taken place in mixtures of certain reactive dyes [28].

2.4.2 Thin Layer Chromatography

In thin layer chromatography (TLC), separation occurs on a flat, solid plate that is coated with a thin layer of silica or alumina. The plate is spotted with a sample, the position is marked with a pencil, and then the plate is placed in a closed solvent chamber where the

solvent is touching the bottom of the plate. Similar to elution in liquid chromatography, the sample is then carried through the stationary phase (plate coating) by the eluent (solvent) which travels up the plate, and allows for components in the sample to separate [2-3]. For each component separated, the retention factor (R_F) is calculated according to Equation A where d_R is the distance from the sample origin to the center of the spot or where maximum intensity occurs on the sample spot, and d_M is the distance from the sample origin to the solvent front [3].

$$(A) \quad R_F = d_R / d_M$$

Benefits of TLC include speed and low cost of equipment and supplies. These factors may explain why this technique is still popular, despite the technological advances of other techniques like HPLC and CE [2-3]. The two main types of plates in TLC are conventional and high performance plates. High performance plates are typically thinner with smaller particle sizes, and can theoretically give smaller R_F than that of conventional plates, but are limited in sample capacity [3]. Both types of plates have been utilized for analysis of dyes.

Natural dyes such as turmeric and indigo blue along with their fabric extracts were analyzed by Bhattacharyya using TLC. Their R_F values were compared and it was shown that the natural dye samples and those extracted from fabrics have very similar if not identical values [27]. Sokolowska-Gadja utilized TLC to monitor the progress of reactions utilized to synthesize disperse dyes and cationic dyes from acid dyes [29]. Konstantinova [19] used TLC to separate and monitor the synthesis of bifunctional monochlorotriazine reactive dyes,

while Koprivanac [30] monitored reactive dyes during the dyeing process in order to produce a method that is more environmentally friendly. Bhattacharyya developed a high performance thin layer chromatographic technique to monitor the alkaline hydrolysis of reactive dyes [27].

2.4.3 Capillary Electrophoresis

Capillary electrophoresis is a technique that allows ions to be separated based on their migration under an electric field. One of the major benefits of CE is the superior resolution obtained over the packed columns of HPLC. Another benefit is the low sample volume needed in CE. This is helpful, especially when dealing with samples with low analyte concentration [1, 31-32]. Croft and Hinks examined different types of dyes such as acid, basic, and reactive dyes by CE. It was found that CE gave superior resolution for the analysis of these dyes, when compared with HPLC [32]. Hansa analyzed a group of five reactive dyes using HPLC and HPCE via UV detection. Results showed superior resolution by HPCE of the reactive dyes, while HPLC exhibited co-elution of a blue and green dye. However, further studies would be needed to improve reproducibility of HPCE runs and for hydrolysis to be successfully conducted under HPCE [31].

2.4.4 High Performance Liquid Chromatography

High pressure or high performance liquid chromatography is helpful for analyzing samples that are thermally unstable or not volatile. For natural dyes, according to Bhattacharyya, HPLC is one of the most efficient analysis methods. Natural dyes were

analyzed on an octadecylsilica (C-18) column using a photodiode array detector (PDA). Results showed the analysis of several dyes including identification of a mixture of manjishtha with alizarin. This natural mixture was very similar to a mixture of dyes analyzed by extraction [27]. Kanazawa measured the water soluble products and precipitates of the degradation of Acid Orange 10 via HPLC [14].

For reactive dye hydrolysis, HPLC is often the technique of choice and ion-pair chromatography is often utilized to improve the analysis. Smith measured the hydrolysis of a commercial sample of Reactive Violet 5 with and without the use of ultrasound energy using HPLC. The use of ultrasound energy was found to increase the rate of dye hydrolysis, which is shown in the chromatograms by the dramatic rise of the hydrolyzed dye peak [16]. Hydrolysis of several reactive fluorotriazine dye mixtures was also measured using HPLC with and without fabric samples. Zotou found that the absence of the fabric produced different hydrolysis behavior in all of the different mixtures between the 60 to 90 minutes time frame, while the presence of the fabric gave the same hydrolysis behavior throughout the dyeing process [13]. Klančnik utilized HPLC to measure the hydrolysis kinetics of several fiber reactive dyes in HPLC [17]. The kinetics of hydrolysis and methanolysis of a reactive dye [9], as well as the influence of pH on these processes were also examined [19].

HPLC can also be utilized with on-line methods (2.4.4.1) and HPLC-MS (2.4.4.2) for analysis of different dyes.

2.4.4.1 On-line HPLC

On-line HPLC methods, particularly for reactive dyes, are of interest, because of the ability to monitor the textile dyeing process [15]. For this to occur, HPLC can be coupled

with other techniques, such as flow injection analysis, sequential injection analysis, immunoextraction, and infrared spectroscopy.

Flow injection analysis (FIA) allows for a sample to be injected into a constantly moving non-segmented carrier stream and transported to a detector, such as a spectrophotometer [33]. Wallace coupled FIA with HPLC in order to monitor dye exhaustion and hydrolysis on-line. This system can be utilized to help determine the most efficient method of dyeing with a reactive dye [1,33]. Sequential injection analysis (SIA) is similar to FIA, but SIA utilizes a selection valve to measure the volumes of carrier stream and sample in the system. Draper conducted a comparison study with SIA and FIA to monitor batch dyeing processes. It was found that both systems are comparable based on precision, accuracy, and monitoring capabilities with SIA having a slight advantage of the ability to adjusting sample size easily, though more sample is required for each measurement [33]. Further studies in this area could lead to a SIA-HPLC method similar to that of the FIA-HPLC.

Another aspect of textile wet processing is waste management. Bouzige utilized on-line immunoextraction with HPLC to measure the trace amounts of dyes and their intermediates in surface water and other industrial waste. The on-line immunoextraction involved utilizing a pre-column that was packed with an immunosorbent, which was specifically designed to target certain types of compounds, such as those which would be found in certain dyes and their intermediates. The major benefit of this technique is the ability to directly link it to HPLC on-line without utilizing exhaustive solid phase extraction (SPE) methods for the sample [34]. Rehorek was also concerned with detoxification of

textile wastes and used a bioreactor coupled with a dual HPLC system to monitor the degradation of an azo dye [35]. Jones examined the degradation of dyes such as Reactive Blue 74 and Acid Blue 25, as well as pesticides with the assistance of HPLC-FTIR [9].

2.4.4.2 LC-MS

HPLC coupled with mass spectrometry is one of the most popular analytical techniques used today, due to its capability of analyzing a wide variety of samples very efficiently. Particularly in the area of biochemistry, where samples often include analytes with highly complex structures, chromatographic separation alone is not completely sufficient, since peak determination is very difficult for such samples. Therefore, the addition of a mass spectrometer as a detector allows for fairly quick determination of structure. LC-MS is heavily used in pharmacokinetics to analyze compounds like nucleic acids and proteins [5], but is also a proven technique for the analysis of dye structures. However, just as HPLC methods can vary for different types of dyes, the method of mass spectrometry used can vary and as such, the HPLC coupled with it would utilize different methods that would reflect the ionization technique.

For natural dyes that are found in historical textiles, analysis of these dyes by LC-MS can be somewhat difficult due to the harsh extraction methods. Zhang developed a method using ethylenediaminetetracetic acid (EDTA) and formic acid, which is a milder extraction technique than using HCl. This allowed for the extracted dyes to be analyzed by LC-MS, without destroying glycosidic linkages in the cellulose [8]. For sulfonated dyes, Holčapek found that these dyes were best analyzed by LC-MS using electrospray mass spectrometry

[7]. However, this method poses a problem when dealing with ion pairing agents, because they can suppress the signal of the mass spectrometer, and can be the source of contamination. A variety of different ion-pairing agents were examined for this effect, and it was found that dialkylammonium acetates and trialkylammonium acetates are the best ion-pairing agents to use to minimize signal suppression [6]. This is particularly important, since dyes analyzed by ion-pair chromatography often used tetraalkylammonium agents, so adjustments would need to be made for such dyes to be examined using the coupled electrospray mass spectrometry technique. For reactive dye analysis, Van Cott was able to utilize HPLC coupled with matrix assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) for analysis of dichlorotriazine dyes in optical films [36].

3. EXPERIMENTAL METHODS AND PROCEDURES

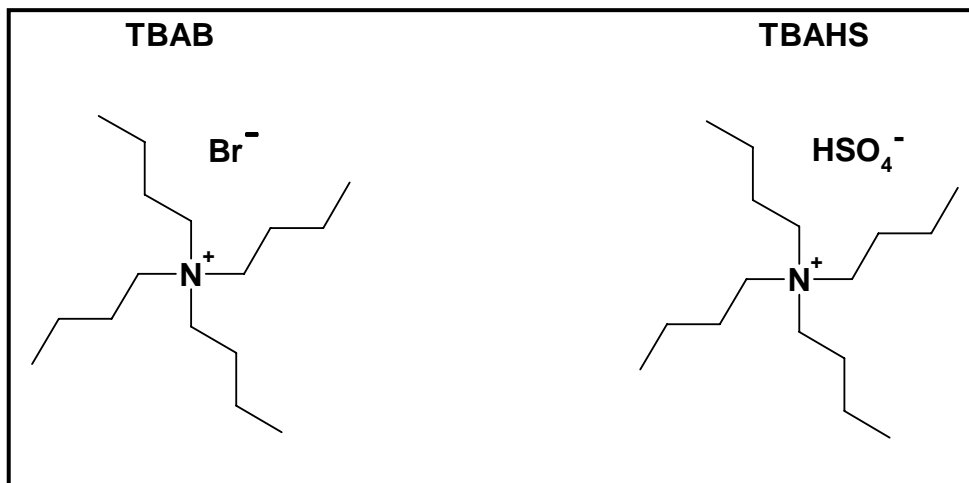
3.1 Materials

Materials used in this study included mobile phase components, dyes, a variety of HPLC columns, and chemicals for experiments.

3.1.1 Mobile Phase Components

The mobile phase for these experiments consisted of an organic phase containing an ion pairing agent (IPA) and a mixed phase that included some of the first organic phase combined with an aqueous phase that contains a buffer. HPLC grade acetonitrile (AN) was obtained from Acros Organics and the 18M ohm water used was obtained from a US Filter Purelab Plus system. Ion pairing agents were tetrabutylammonium bromide (TBAB) from Acros Organics, and tetrabutylammonium hydrogen sulfate (TBAHS) from Fluka Chemika. ACS grade monobasic ammonium phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) was obtained from Fisher Chemicals. Their structures are shown in Figure 3.1 below.

Figure 3.1: Ion-Pair Reagents



3.1.2 Mobile Phase Preparation

The mobile phases were prepared in 1-L increments for both phase A (mixed phase) and phase B (organic phase). TBAB (10.477g, 0.025 mol) or TBAHS (11.035g, 0.025 mol) was added to 1.30L of AN and stirred to make mobile phase B. $\text{NH}_4\text{H}_2\text{PO}_4$ (4.026g, 0.050 mol) was added to 0.70L of H_2O and stirred. A 0.30-L portion of phase B was added to the $\text{NH}_4\text{H}_2\text{PO}_4$ buffer and further stirred to generate mixed phase A. These two solutions were filtered using 0.22 μm , 47mm MAGNA Nylon filters (Fisher Chemical) and placed in the solvent reservoirs of the HPLC system. The organic phase was placed in reservoir B, and the mixed phase was placed in reservoir A.

3.1.3 Dyes

All of the dyes used in this study were commercial. Table 3.1 shows the dyes' commercial and colour index (C.I.) names. All of the dye solutions for analysis were

prepared by placing 30 mg of dye in a volumetric flask and diluting to 100.00mL with HPLC grade water. To prepare for HPLC analysis, each dye solution was filtered using 0.2µm, 17mm PVDF syringe filters obtained from Alltech Associates Inc.

Table 3.1: Commercial and C.I. Names of Dyes

Colour Index Name (C.I.)	Commercial Name
Reactive Red 43	Cibacron Scarlet 2G-E
Reactive Red 180	Evermax Red SF-3B
Reactive Red 239	Remazol Brilliant Red 3BS-A
Reactive Yellow 3	Cibacron Yellow R
Reactive Yellow 135	Procion Yellow H-E6G
Reactive Yellow 168	Cibacron Yellow C-R-01
Reactive Blue 21	Remazol Turquoise G-A
Reactive Blue 52	Cibacron Blue TR-E
Reactive Blue 238	Cibacron Navy C-B

Figures 3.2, 3.3, 3.4, 3.5, 3.6, and 3.7 are the structures of Reactive Red 43, Reactive Red 180, Reactive Red 239, Reactive Yellow 3, Reactive Yellow 135, and Reactive Blue 238 respectively. Reactive Blue 21 is shown in Figure 3.8.

Figure 3.2: Structure of Reactive Red 43

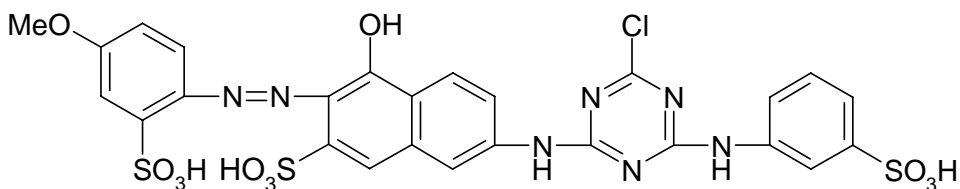


Figure 3.3: Structure of Reactive Red 180

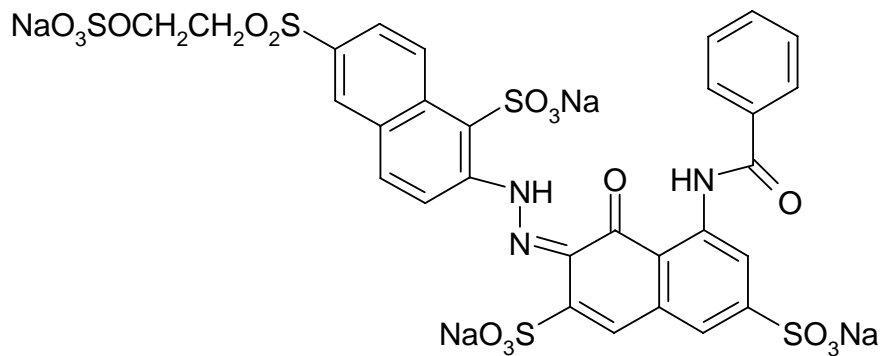


Figure 3.4: Structure of Reactive Red 239

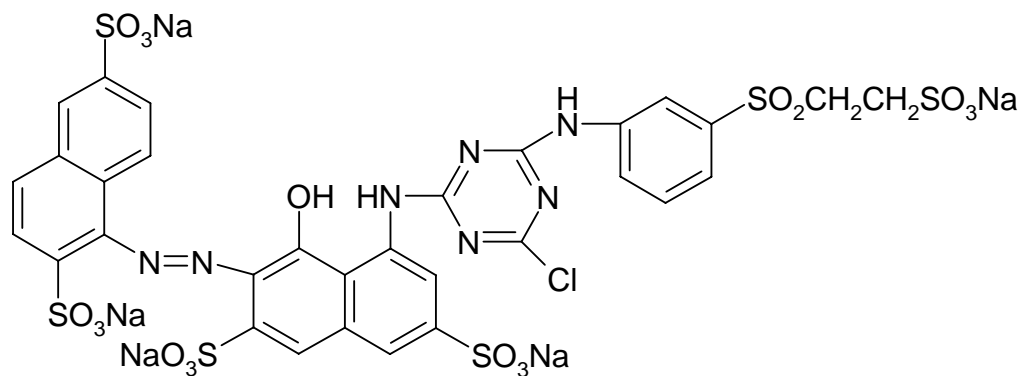


Figure 3.5: Structure of Reactive Yellow 3

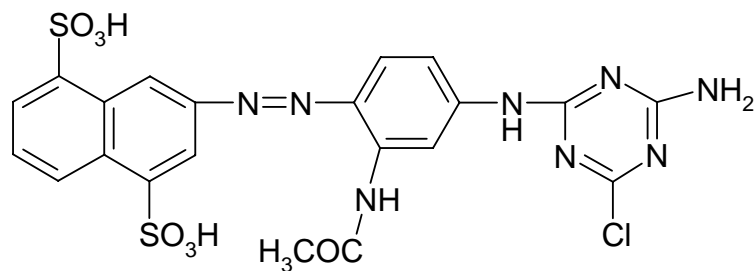


Figure 3.6: Structure of Reactive Yellow 135

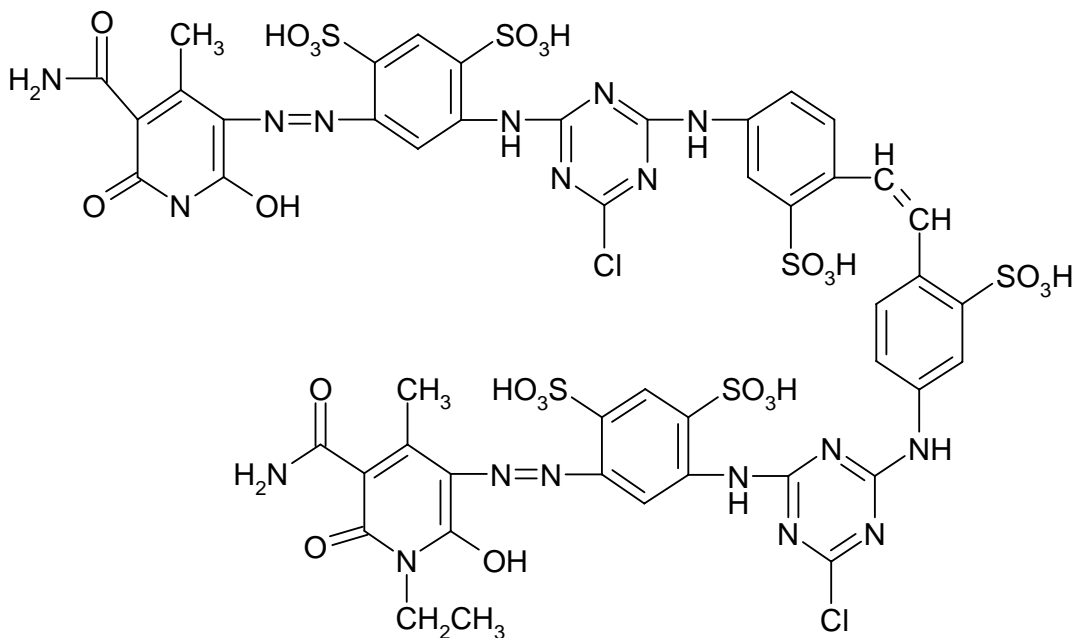
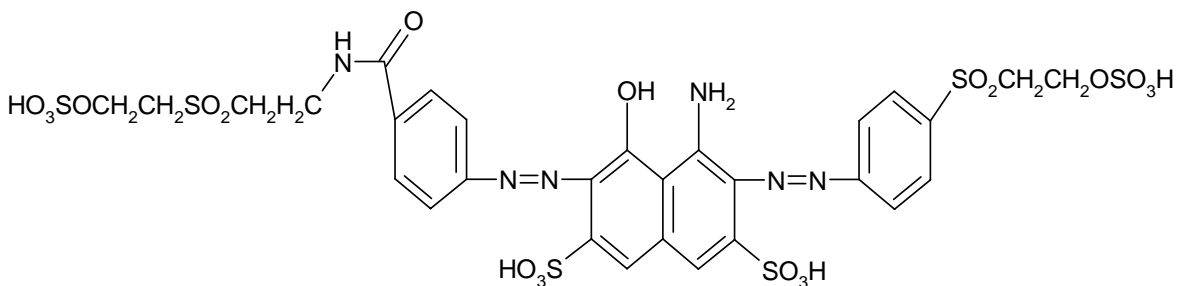


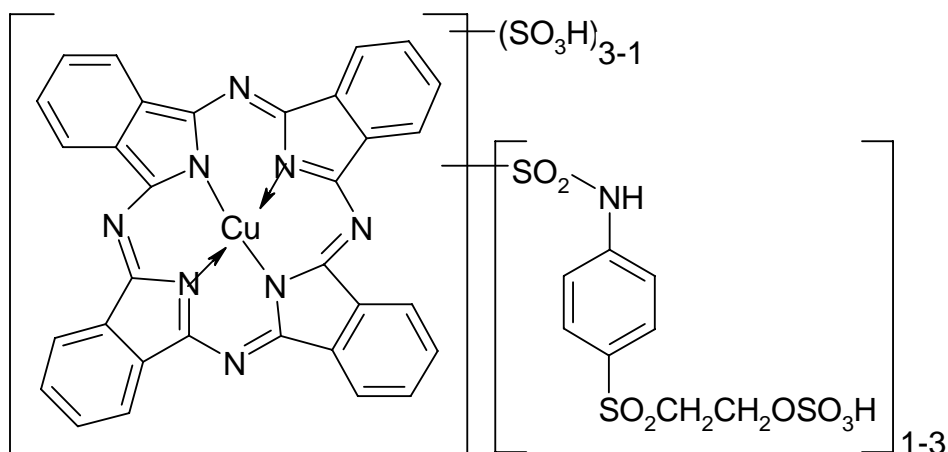
Figure 3.7: Structure of Reactive Blue 238



Reactive Yellow 3, Reactive Yellow 135, and Reactive Red 43 are monochlorotriazine dyes, while Reactive Red 180 is a vinylsulfone dye. Reactive Blue 238 has two sulfatoethylsulfone groups, so they are bireactive dyes, since they contain two reactive groups. Reactive Red 239 contains a monochlorotriazine group and a

sulfatoethylsulfone group, so it is a heterobireactive dye, or a dye that contains two different reactive groups. Bireactive dyes are important, because they can increase the amount of dye fixed to the fabric, and thus decreasing the amount of dye wasted [21]. Reactive Blue 21 is a copper-complex reactive dye with sulfatoethylsulfone groups. A general structure of Reactive Blue 21 can be seen in Figure 3.8 below.

Figure 3.8: Structure of Reactive Blue 21



3.1.4 Chromatographic Columns

The columns utilized in this study are listed in Table 3.2, which includes the name of the column, type of stationary phase, its dimensions (length and inner diameter), particle size, manufacturer, and designation for cartridge columns. Those columns denoted as cartridge columns also contained guard cartridges within the cartridge. These guard cartridges are shown in Table 3.3 below along with their name, dimensions, particle size and manufacturer. The columns underwent a 30-minute water wash at 1mL/min before the mobile phase was

introduced to the stationary phase. When all analytical runs were completed on a given column or at the end of the day, the column was subjected to another 30-minute water wash at the same flow rate to ensure that the buffer salts were completely removed from the column. That wash was followed by a 15-minute rinse at 1mL/min with either 100% acetonitrile or the storage solvent. Prior to the washing, these solvents were vacuum filtered.

Table 3.2: Columns for HPLC Analysis

Column	SP	L (mm)	I.D. (mm)	Particle Size (um)	Manufacturer	Cartridge (Y or N)
Adsorbosphere	C18	250	4.6	5	Alltech	N
Alltima	C6H5	250	4.6	5	Alltech	N
Atlantis dC18	C18	150	3.9	3	Waters	N
Exsil Butyl 300A	C4	250	4.6	5	Alltech	Y
Exsil ODS	C18	250	4.6	5	Alltech	Y
Exsil TMS 100A	C1	250	4.6	5	Alltech	Y
Nova-Pak C18 60A	C18	150	3.9	4	Waters	N
Symmetry	C18	150	4.6	5	Waters	N
Zorbax Reliance	C6H5	40	4.6	3	MAC-MOD	Y

Table 3.3: Guard Cartridges

Guard Cartridge	L (mm)	I.D.(mm)	Particle Size (um)	Manufacturer
Exsil Butyl 300A	10	4.6	5	Alltech
Exsil ODS	10	4.6	5	Alltech
Exsil TMS 100A	10	4.6	5	Alltech
Zorbax	12.5	4.6	5	MAC-MOD

3.1.5 Chemicals for Experiments

Reactive Blue 21 was examined following its hydrolysis, as well as by exhaust dyeing and pad batch dyeing. The pH paper used in these experiments was obtained from Fisher Scientific, and the fabric used in the dyeing experiments was a white woven cotton fabric, that was desized, scoured, and bleached. For hydrolysis experiments, HCl and NaOH were obtained from Aldrich Chemical. Na_2CO_3 and $\text{Na}_2\text{SO}_4 \cdot 10 \text{H}_2\text{O}$ for the exhaust dyeing were both certified ACS grade and were obtained from Fisher Scientific. Penetrant EH, a wetting agent, was obtained from Clariant, while the 42°Be Na_2SiO_3 and 50% NaOH were obtained from Fisher Scientific for the pad batch dyeing.

3.2 Instrumentation

3.2.1 UV-Visible Spectroscopy

For UV-Visible analysis, all dyes were diluted at least 1 to 10 from the standard stock solution of 30mg of commercial dye diluted to 100.00mL in water. A Cary 3E UV-Visible (Varian, Inc.) spectrophotometer was used with Cary WinUV software operated in Scan mode. The lambda max (λ_{max}) of the dyes was obtained and used to select wavelength of analysis in HPLC.

3.2.2 High Performance Liquid Chromatography System (HPLC)

Two HPLC systems were utilized in this study. The first system used was a Breeze instrument, but the majority of the experiments were performed on an Alliance system.

3.2.2.1 Breeze HPLC System

The Breeze chromatographic system consisted of a Waters 1525 Series binary HPLC pump system with the Waters 2487 Dual Absorbance Detector and Waters 2414 Refractive Index Detector. Samples were introduced into the system via a Rheodyne 7725i injector with a 20- μ l sample loop. The loop was loaded by manual injection using a Hamilton 100- μ l syringe. Data collection, storage, and peak analysis were performed by the Breeze Version 3.2 on a Dell Dimension 4100 computer workstation.

3.2.2.2 Alliance HPLC System

The Alliance chromatographic system was operated through a Dell Optiplex GX270 workstation. The Waters Alliance 2695 Separations Module system consisted of a four-bottle solvent reservoir, solvent management system, Performance Plus inline degasser, and an autosampler. Analyte detection was performed using two systems which consisted of a setup of two of the following detectors: Waters 2487 Dual Absorbance Detector, Waters 2414 Refractive Index Detector, and an updated 2996 Waters Photodiode Array detector. System 1 used the 2487 detector and the 2414 refractive index detector, while System 2 consisted of the 2996 PDA and the 2414 refractive index detector. Samples were introduced to the column via automatic injection through a 250- μ l syringe into a 100- μ l sample loop, where 10 μ l of sample was injected onto the column. Data collection, storage, and peak analysis were performed by Empower Pro software.

3.3 Experiments

3.3.1 Ion-Pair Reagents (IPA)

Two ion-pair reagents (IPA), tetrabutylammonium bromide (TBAB) and tetrabutylammonium hydrogen sulfate (TBAHS), were examined to determine which is more suitable for the analysis of reactive dyes by HPLC. The experiment was conducted on the Alliance System 1. The basic mobile phase used for the runs consisted of the two mobile phases listed in Figure 3.9, where IPA represents the ion-pairing agent used.

Figure 3.9: Ion-Pair Reagent Mobile Phase

Mobile Phase A: 30% of MP B/70% H₂O + 0.05M NH₄H₂PO₄

Mobile Phase B: AN + 0.025M IPA

The gradient used to compare the ion pair reagents is denoted as Gradient 1 and this method is shown below in Table 3.4.

Table 3.4: Gradient 1

Flow (mL/min)	Time	A	B
1	Initial	70	30
1	1.5	50	50
1	3.5	70	30

This mobile phase gradient is based on the method of Smith and Thakore with some adjustments [23]. The order of the mobile phases was switched in order to reflect the standard in high performance liquid chromatography, where mobile phase A is the most aqueous mobile phase. The flow rate was reduced, and the gradient was shortened.

Three different columns (Symmetry, Novapak, and Atlantis) were used to compare the ion-pair reagents. All of the columns were packed with a C₁₈ stationary phase and were the same length, but varied in inner diameter and particle size. Table 3.5 shows the dyes analyzed by the two IPA mobile phases and the columns used for analysis.

Table 3.5: Columns and Dyes Examined by Ion-Pair Reagents

Dyes	Symmetry	Novapak	Atlantis
Reactive Red 180		X	
Reactive Red 231	X	X	
Reactive Yellow 3	X	X	
Reactive Yellow 135	X		
Reactive Yellow 168	X	X	
Reactive Blue 21	X	X	X

3.3.2 Stationary Phases

3.3.2.1 Stationary Phases

Stationary phases were examined to determine which phase provided optimal analysis of reactive dyes. The stationary phases of interest included C₁, C₄, C₁₈, and phenyl. Several columns with different C₁₈ stationary phases were examined. The columns used have been

previously listed in Table 3.2.

3.3.2.2 Gradient Adjustments

Gradient 1 was previously shown in Table 3.4. The gradient used in the Breeze system is shown in Table 3.6, and is referred to as Gradient 2.

Table 3.2: Gradient 2

Flow (mL/min)	Time	A	B
1	Initial	70	30
1	5.0	50	50
1	13.0	70	30

Gradient 2 was also utilized for the C₁₈ and C₄ cartridge runs on the Alliance HPLC System

1. Since Reactive Blue 21 has a more complex structure than the other dyes, further adjustments were made to the gradient to optimize analysis of this particular dye. In Table 3.7, a preferred method for the analysis of Reactive Blue 21 is shown.

Table 3.7: Optimized Gradient for Reactive Blue 21

Flow (mL/min)	Time	A	B
1	Initial	70	30
1	10	50	50
1	12	70	30

3.4 Reactive Blue 21 Hydrolysis

Hydrolysis of Reactive Blue 21 and two different types of dyeings, exhaust dyeing and pad batch dyeing, were carried out to determine if any of the peaks in the dye could be assigned as sulfatoethylsulfone (SES), vinyl sulfone (VS), or hydroxyethylsulfone (HES).

3.4.1 Hydrolysis

Reactive Blue 21 was prepared by placing dye (100 mg) in a volumetric flask and diluting with HPLC water to 100.00mL. A 5g/L NaOH solution was made by adding HPLC water (0.10L) to NaOH (0.5g, 20-40 mesh beads), while an HCl solution was prepared by adding 37% HCl (0.25mL) to 100mL of HPLC water. The dye solution (50mL) was added to the NaOH solution (50mL) in a beaker and placed in a heating bath. Once the temperature of the dye mixture reached 65°C, a 1-mL aliquot was taken, followed by aliquots every five minutes for an hour. Each aliquot was neutralized by the HCl solution to pH 7 (pHydrion papers (1 to 12)) and placed on ice. These samples were diluted to 5.00mL with HPLC water, filtered with PVDF syringes, and analyzed by HPLC.

3.4.2 Exhaust Dyeing

The exhaust dyeing was conducted on an Ahiba 1000 Texomat machine. Na_2CO_3 (20g) was added to 100mL of hot H_2O in a 250-mL beaker. Water (100mL) was heated to 30°C and $\text{Na}_2\text{SO}_4 \cdot 10 \text{H}_2\text{O}$ (28g) was added to it. Reactive Blue 21 (0.4g) was added to the salt solution and diluted with water to 389mL. Cotton fabric (20g) was placed in the dye mixture. The first sample (2.0mL) was pipetted from the dye solution, and the Na_2CO_3

solution (2.75mL) was added. A second sample was taken, and the dye solution was heated to 60°C at a rate of 1.5°C/min. After the temperature in the dyebath reached 60°C, the Na₂CO₃ solution (8.75mL) was added, and a third sample was taken. The dyebath temperature was held at 60°C, and 30 minutes later, a fourth aliquot was removed. Subsequently two more samples were taken at ten minute intervals. All extracted samples were immediately neutralized with HCl to pH 7, placed in an ice bath, and later diluted to 5.00mL with water, filtered with PVDF syringes, and analyzed by HPLC.

3.4.3 Pad Batch Dyeing

The dye was applied to 100% cotton fabric on a Werner Mathis AG HVF padder. A bath of Reactive Blue 21 was prepared by adding deionized water (150mL) to dye (20g), and Penetrant EH (0.8g). The first sample (0.5mL) was pipetted from this solution and diluted to 50.0mL in water. An alkali solution was then made by first adding 50% NaOH (5mL) to 42°Be Na₂SiO₃ (10mL) in 20mL of water, and this was diluted with deionized water to 50.00mL. The alkali solution was then added to the remaining dye solution, and this mixture was used to pad a 12'' x 12'' swatch of cotton at 100% wet pick up. The fabric was placed in a plastic bag to dwell overnight, and the next day, the fabric was washed with water, and the water left was then neutralized with HCl to pH 7 and diluted with water to 1.00L. This sample, along with the first sample, was filtered with PVDF syringes, and analyzed by HPLC.

4. RESULTS AND DISCUSSION

4.1 Ion-Pair Experiments

Ion-pair chromatography has been used successfully for the analysis of reactive dyes [13-18, 37]. In order to develop an improved method, two particular ion-pair reagents (TBAB and TBAHS) were examined. The purpose of this experiment was to deduce which of the ion pair reagents would work best for the analysis of these particular reactive dyes. The two ion-pair reagents were compared using Gradient 1 (see Table 3.4) on two different columns (Symmetry, Novapak) for the dyes, while Reactive Blue 21 was examined on an additional column (Atlantis). The dyes analyzed with the ion-pair reagents on a particular column were listed in Table 3.5. Factors used to compare the results obtained using the ion-pair reagents include the number of separated peaks and k' .

4.1.1 TBAB and TBAHS on Symmetry Column

Figures 4.1, 4.3, 4.5, and 4.7 are the chromatograms of the C. I. Reactive Red 239, C. I. Reactive Yellow 3, C. I. Reactive Yellow 135, and C. I. Reactive Yellow 168 on the Symmetry column using TBAB as the ion-pair reagent, while Figures 4.2, 4.4, 4.6, and 4.8 are the same dyes analyzed using TBAHS. All HPLC chromatograms are shown as absorbance versus time.

Figure 4.1: C. I. Reactive Red 239 on Symmetry (C18) Using TBAB and Gradient 1

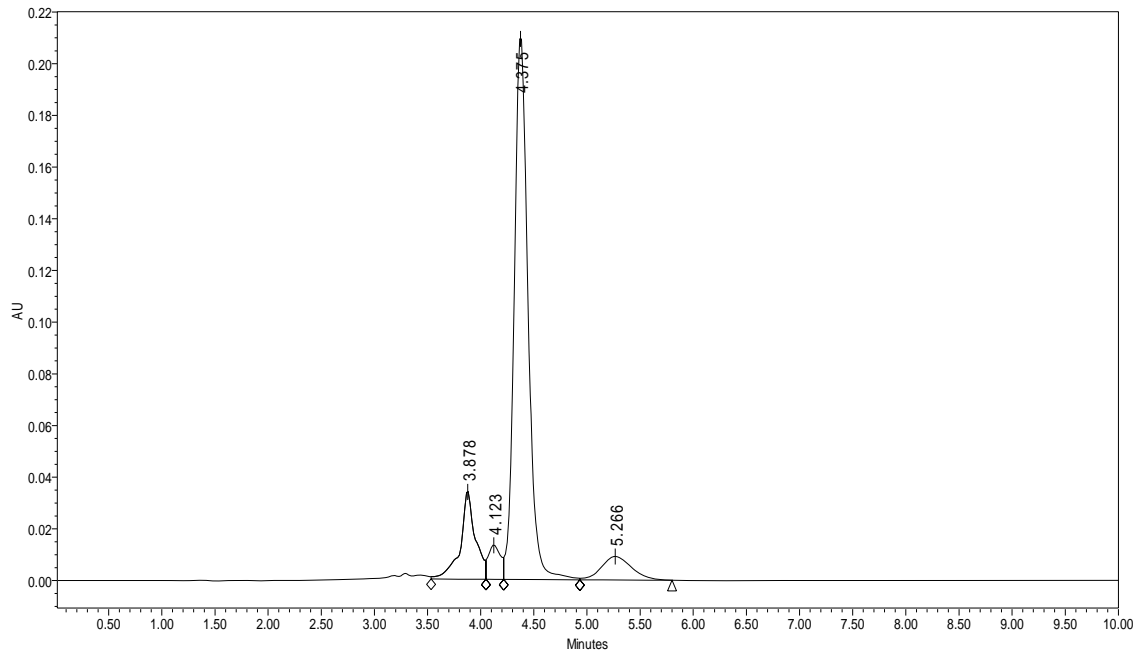


Figure 4.2: C. I. Reactive Red 239 on Symmetry (C18) Using TBAHS and Gradient 1

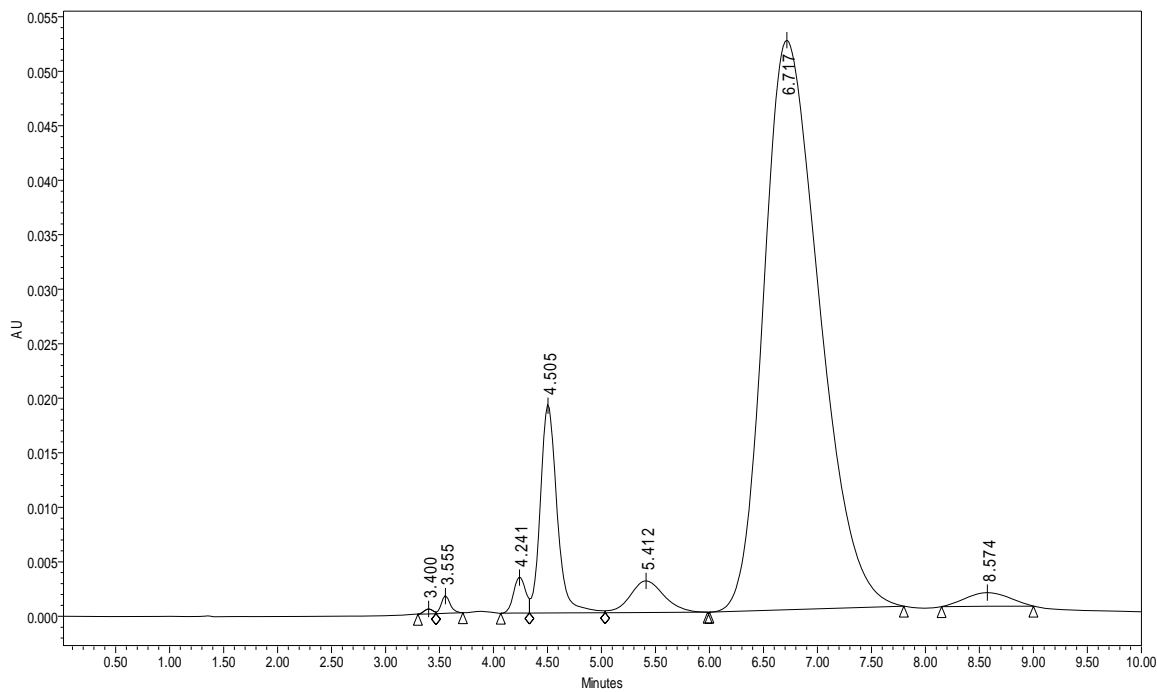


Figure 4.3: C. I. Reactive Yellow 3 on Symmetry (C18) Using TBAB and Gradient 1

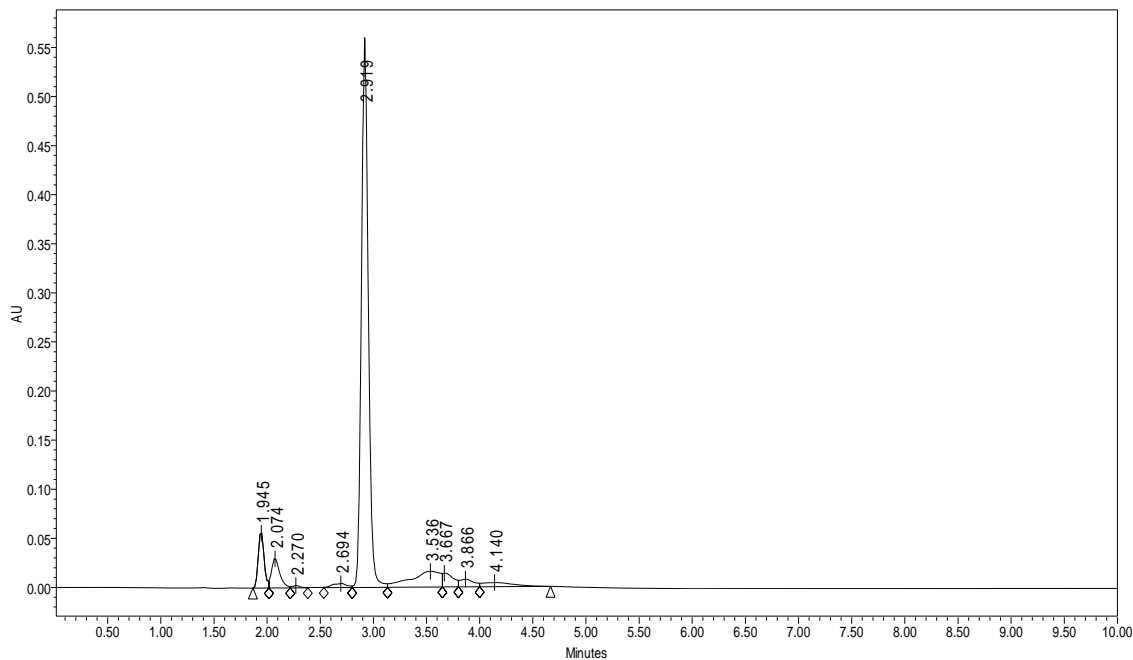


Figure 4.4: C. I. Reactive Yellow 3 on Symmetry (C18) Using TBAHS and Gradient 1

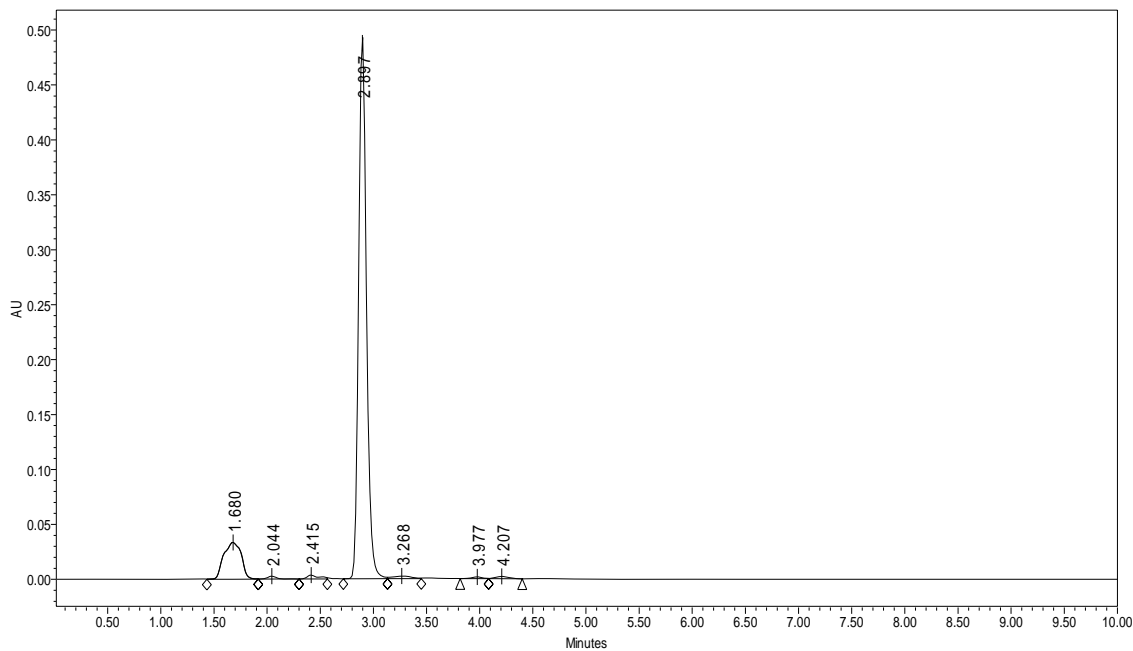


Figure 4.5: C. I. Reactive Yellow 135 on Symmetry (C18) Using TBAB and Gradient 1

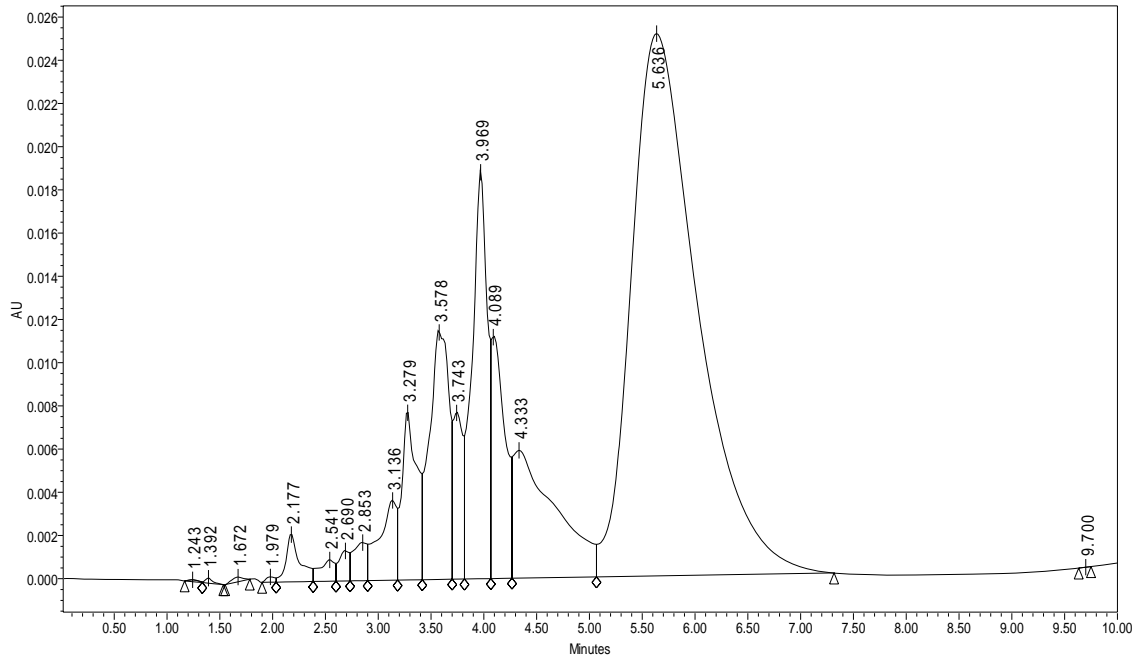


Figure 4.6: C. I. Reactive Yellow 135 on Symmetry (C18) Using TBAHS and Gradient 1

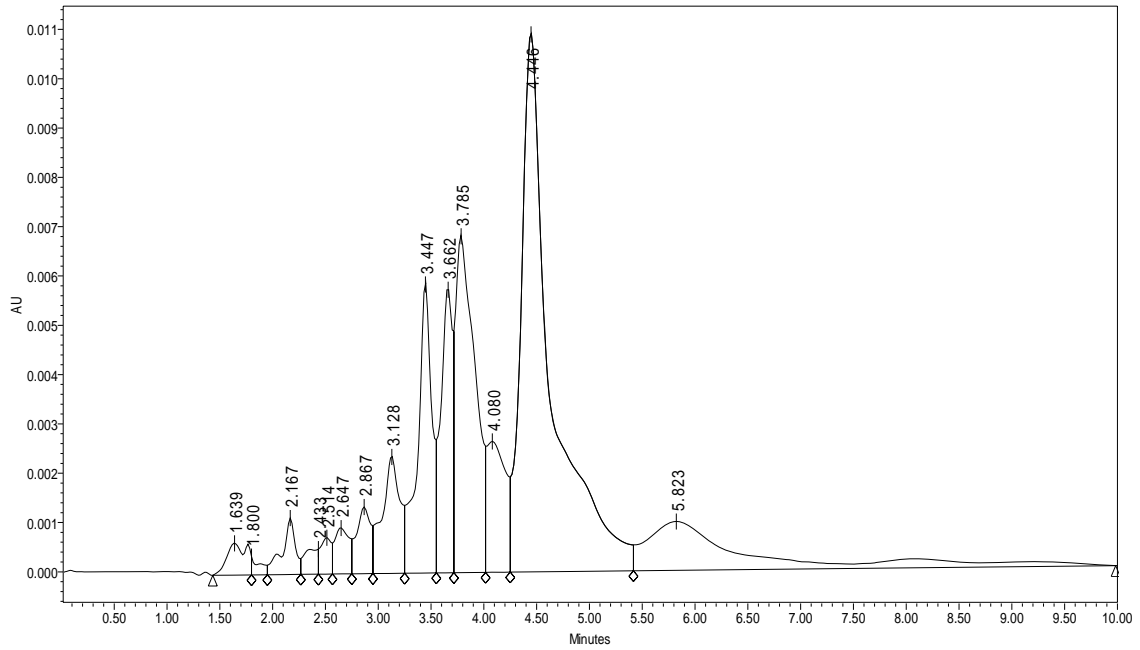


Figure 4.7: C. I. Reactive Yellow 168 on Symmetry (C18) Using TBAB and Gradient 1

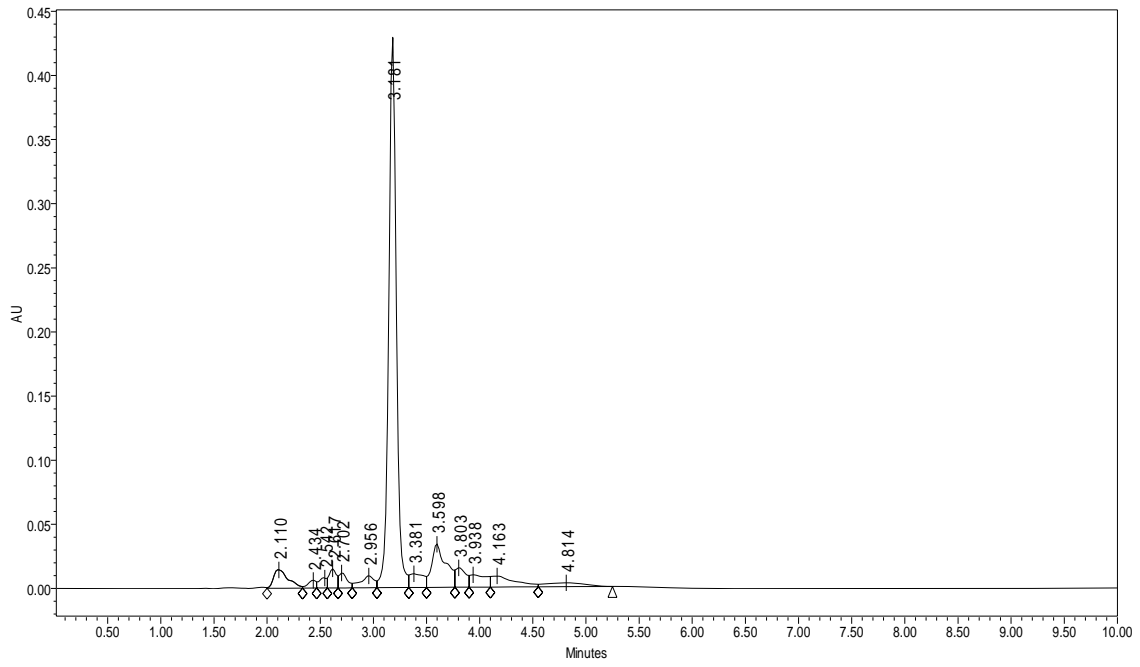
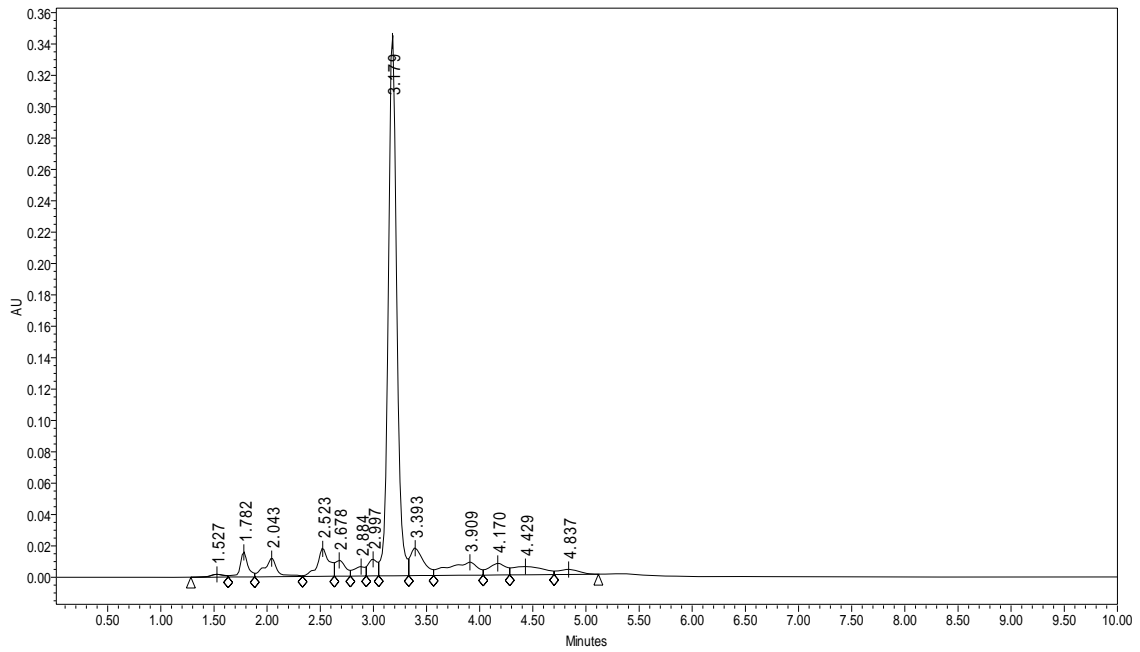


Figure 4.8: C. I. Reactive Yellow 168 on Symmetry (C18) Using TBAHS and Gradient 1



The retention factor is calculated using Equation 4.1, where t is the retention time of the analyte, and t_m is the dead volume, or time needed for a molecule of mobile phase to pass through the column:

$$(4.1) \quad k' = \frac{t - t_m}{t_m}$$

Table 4.1 shows a comparison of number of resolved peaks in the chromatograms, while Table 4.2 shows the retention factor (k') of the main (highest percent area) analyte peak for each chromatogram.

Table 4.1 Number of Resolved Peaks on Symmetry (C18) Column and Gradient 1

Dyes	TBAB	TBAHS	Diff
Reactive Red 239	4	7	-3
Reactive Yellow 3	9	7	2
Reactive Yellow 135	17	14	3
Reactive Yellow 168	13	13	0

Table 4.2 k' Values on Symmetry (C18) Column and Gradient 1

Dyes	TBAB	TBAHS
Reactive Red 239	3.38	5.72
Reactive Yellow 3	1.92	1.90
Reactive Yellow 135	4.64	3.45
Reactive Yellow 168	2.18	2.18

For these four dyes, separation is slightly better using the TBAB as the ion-pair reagent.

With the TBAB, slightly more peaks are resolved when compared with the TBAHS. Both the TBAB and TBAHS yield k' values on average in the ideal range between 2-10 [3].

When comparing the chromatograms of the Reactive Red 239, however, more peaks are

resolved using the TBAHS and their retention factor is higher. This shows that Reactive Red 239 spends more time migrating through the column with TBAHS, but this effect is not necessarily better for separation since peak broadening occurs with this IPA, but not with the TBAB. This same effect is also seen with the Reactive Yellow 135 with TBAB, but not with TBAHS.

4.1.2 TBAB and TBAHS on Novapak Column

Figures 4.9, 4.11, 4.13, and 4.15 are the chromatograms of the Reactive Red 180, Reactive Red 239, Reactive Yellow 3, and Reactive Yellow 168 on the Novapak column using TBAB as the ion-pair reagent, while Figures 4.10, 4.12, 4.14, and 4.16 are the chromatograms for the same dyes using TBAHS.

Figure 4.9: C. I. Reactive Red 180 on Novapak(C18) Using TBAB and Gradient 1

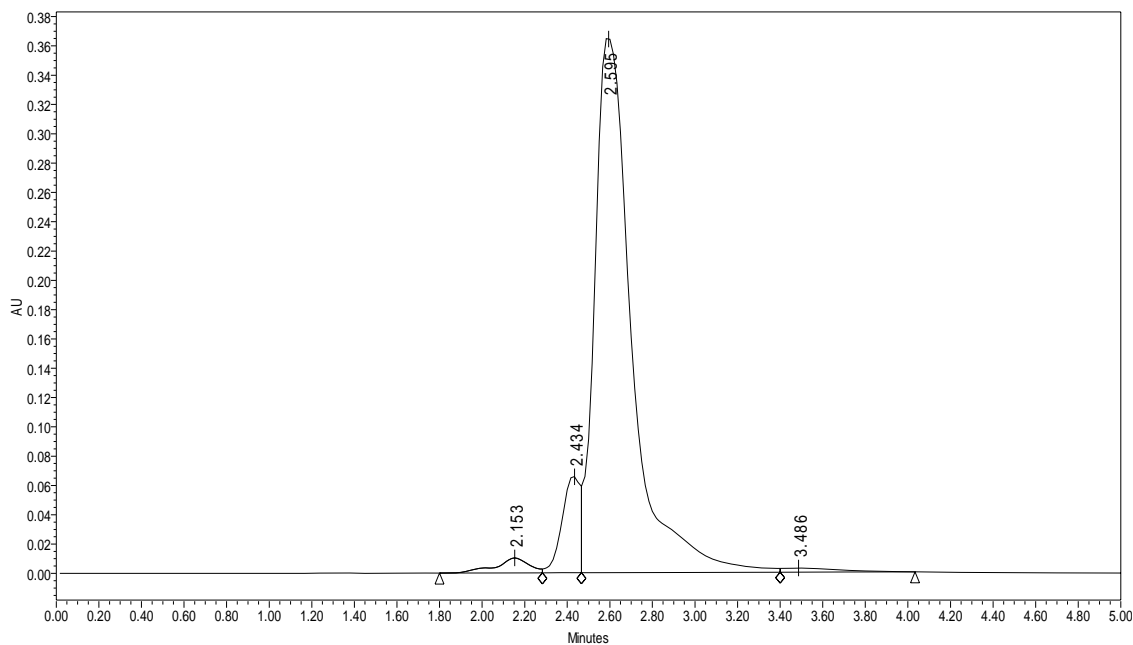


Figure 4.10: C. I. Reactive Red 180 on Novapak(C18) Using TBAHS and Gradient 1

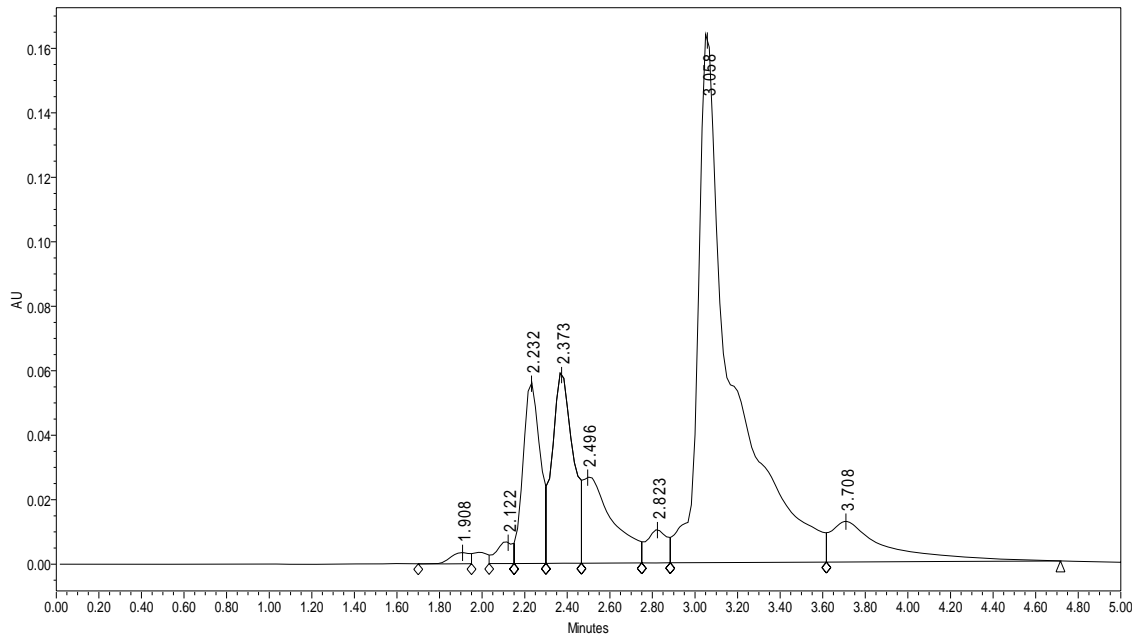


Figure 4.11: C. I. Reactive Red 239 on Novapak(C18) Using TBAB and Gradient 1

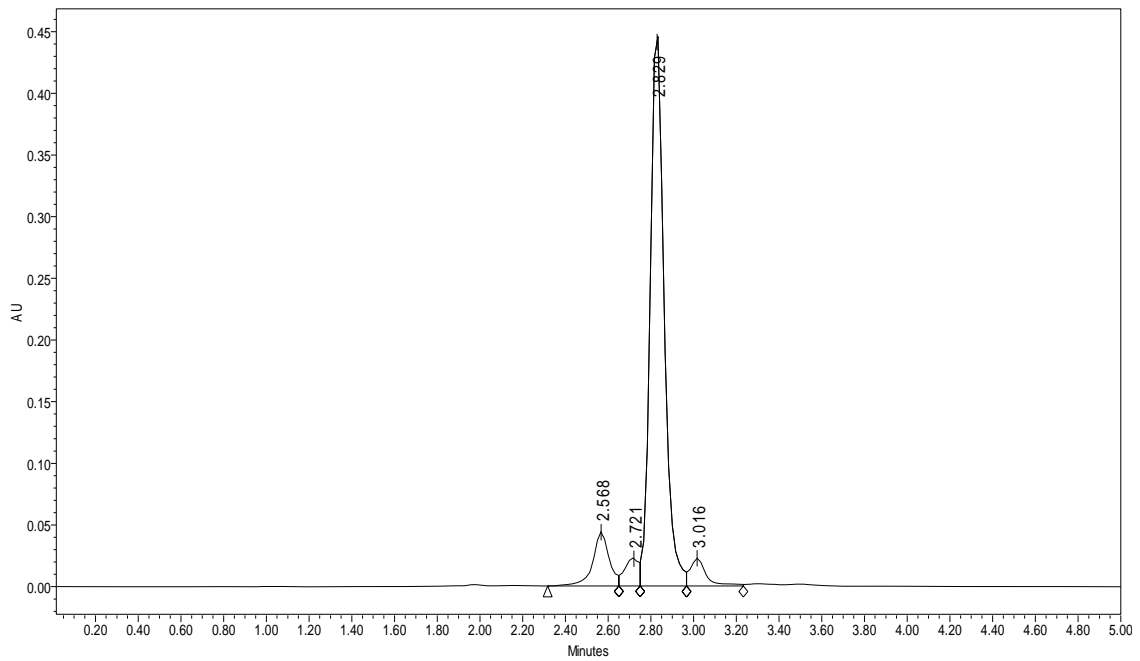


Figure 4.12: C. I. Reactive Red 239 on Novapak (C18) Using TBAHS and Gradient 1

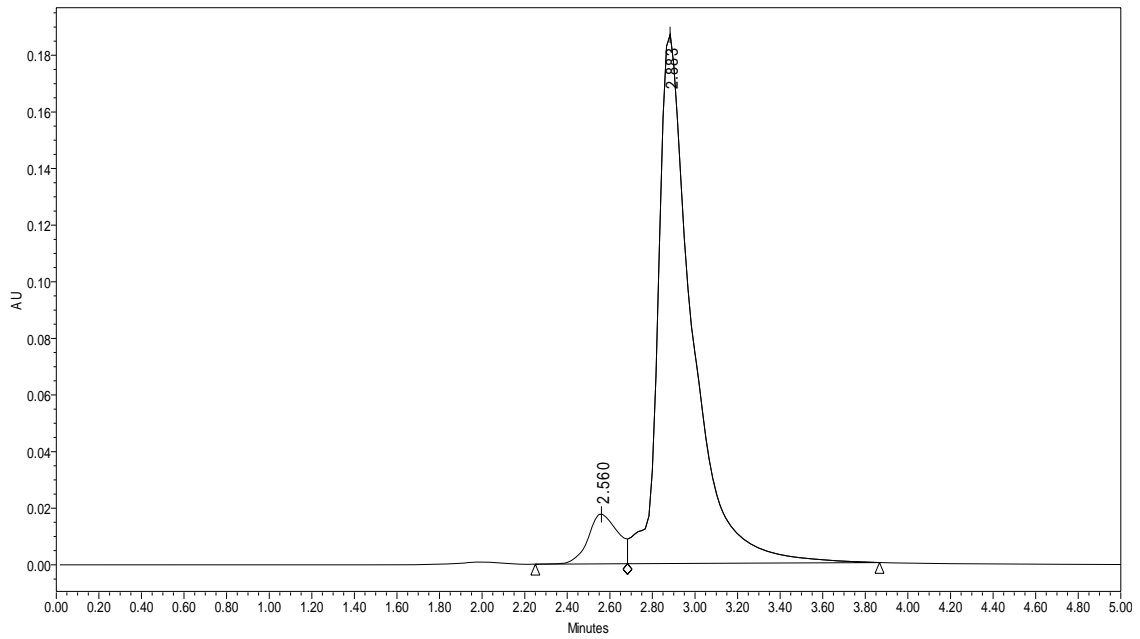


Figure 4.13: C. I. Reactive Yellow 3 on Novapak (C18) Using TBAB and Gradient 1

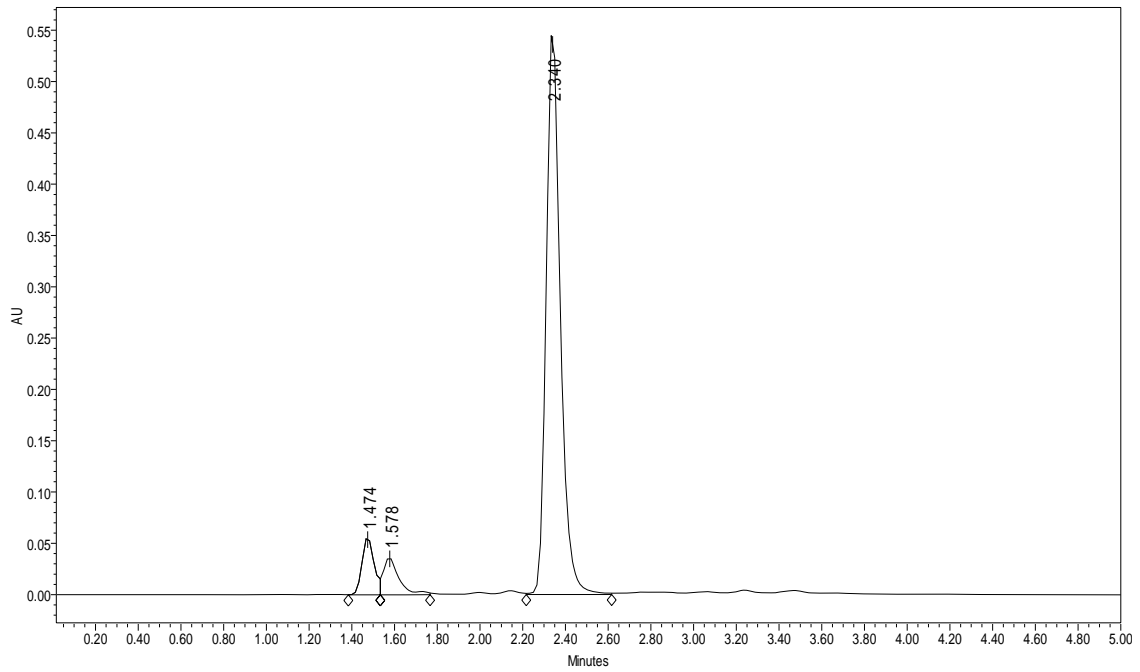


Figure 4.14: C. I. Reactive Yellow 3 on Novapak (C18) Using TBAHS and Gradient 1

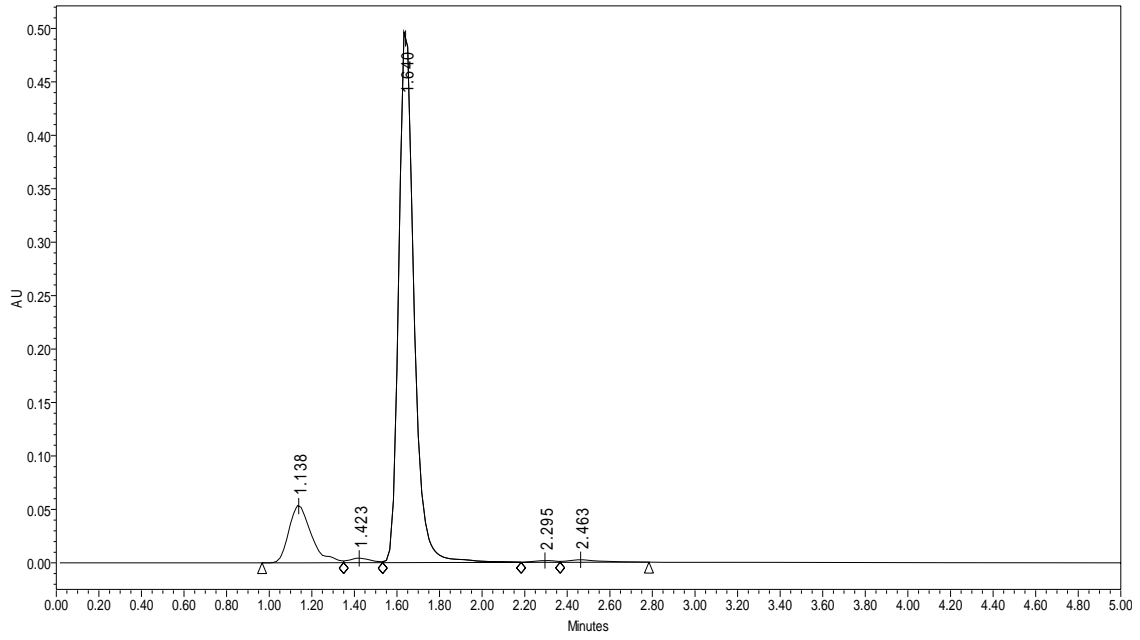


Figure 4.15: C. I. Reactive Yellow 168 on Novapak (C18) Using TBAB and Gradient 1

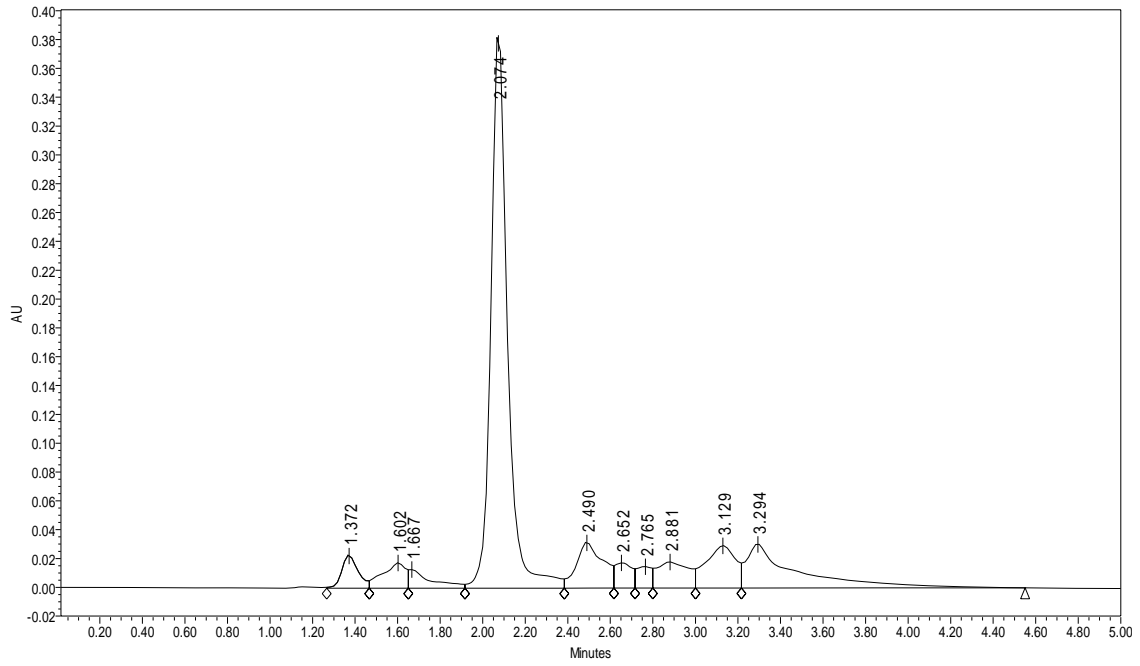
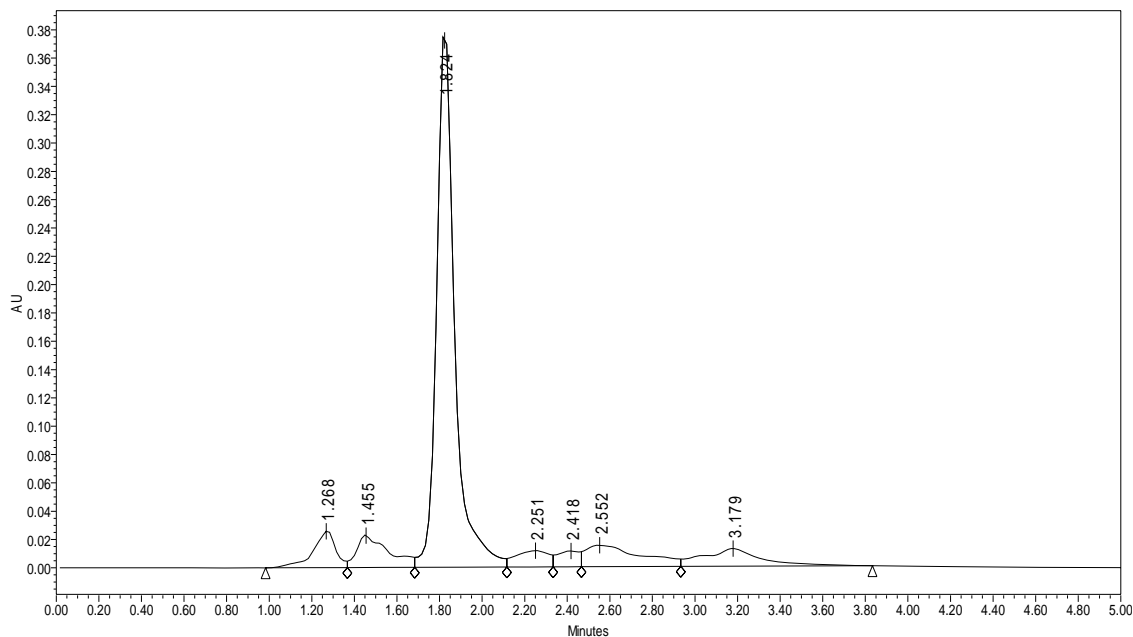


Figure 4.16: C. I. Reactive Yellow 168 on Novapak (C18) Using TBAHS and Gradient 1



For Reactive Red 180, more resolved peaks are obtained by using the TBAHS, but for the other 3 dyes, a comparable or greater number of peaks is seen in the chromatograms with TBAB as the ion-pair reagent. This is also shown in Table 4.3 below. The k' values are higher for the red dyes using the TBAHS than with the TBAB, but for the yellow dyes, TBAB yielded higher values in Table 4.4. Due to the differences that occur with the TBAB and TBAHS on the Novapak column, it is difficult to determine which IPA produced the best result for this column.

Table 4.3 Number of Resolved Peaks on Novapak (C18)Column and Gradient 1

Dyes	TBAB	TBAHS	Diff
Reactive Red 180	4	8	-4
Reactive Red 239	4	2	2
Reactive Yellow 3	3	3	0
Reactive Yellow 168	10	7	3

Table 4.4 k' Values on Novapak (C18)Column and Gradient 1

Dyes	TBAB	TBAHS
Reactive Red 180	1.60	2.06
Reactive Red 239	1.83	1.88
Reactive Yellow 3	1.34	0.64
Reactive Yellow 168	1.07	0.82

4.1.3 Reactive Blue 21 on Atlantis Column

Reactive Blue 21 posed a unique challenge, since the dye did not yield good separation on the Novapak or Symmetry columns. This can be clearly seen in Figure 4.17, which is a chromatogram of the dye with TBAB on the Novapak column, and Figure 4.18 is with TBAHS on the Novapak column. From the chromatograms below (Figures 4.19 and 4.20), one could easily deduce that TBAHS is a better IPA for the analysis of Reactive Blue 21. However since both columns had difficulty separating the dye, it was of interest to see if the dye could be examined on another column.

Figure 4.17: C. I. Reactive Blue 21 on Novapak (C18) Using TBAB and Gradient 1

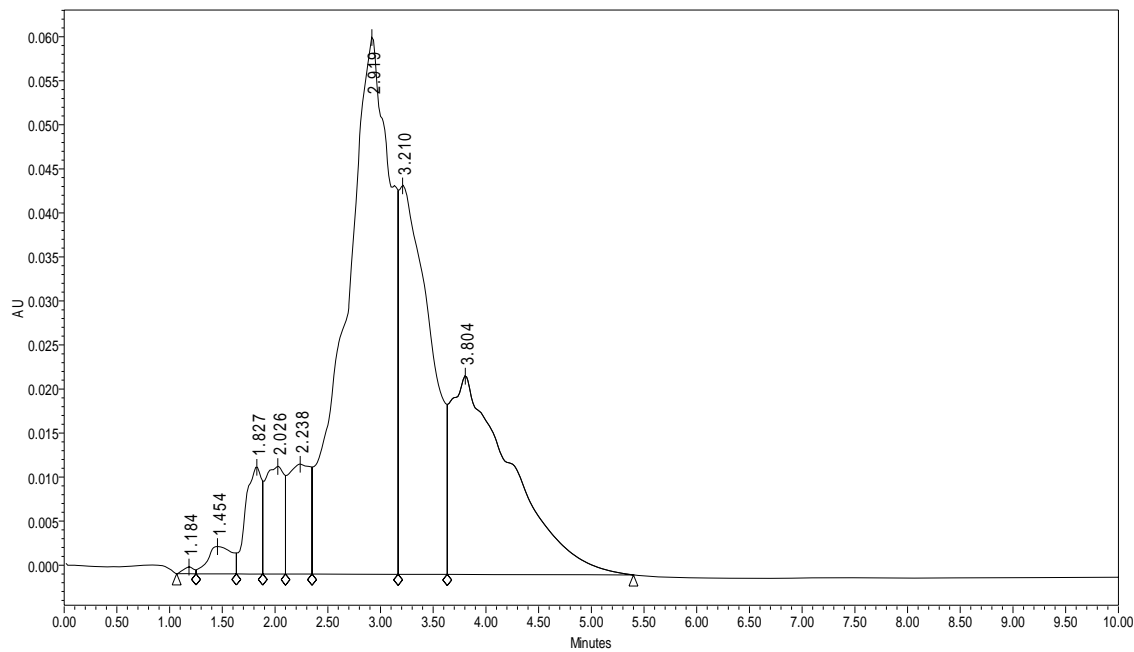
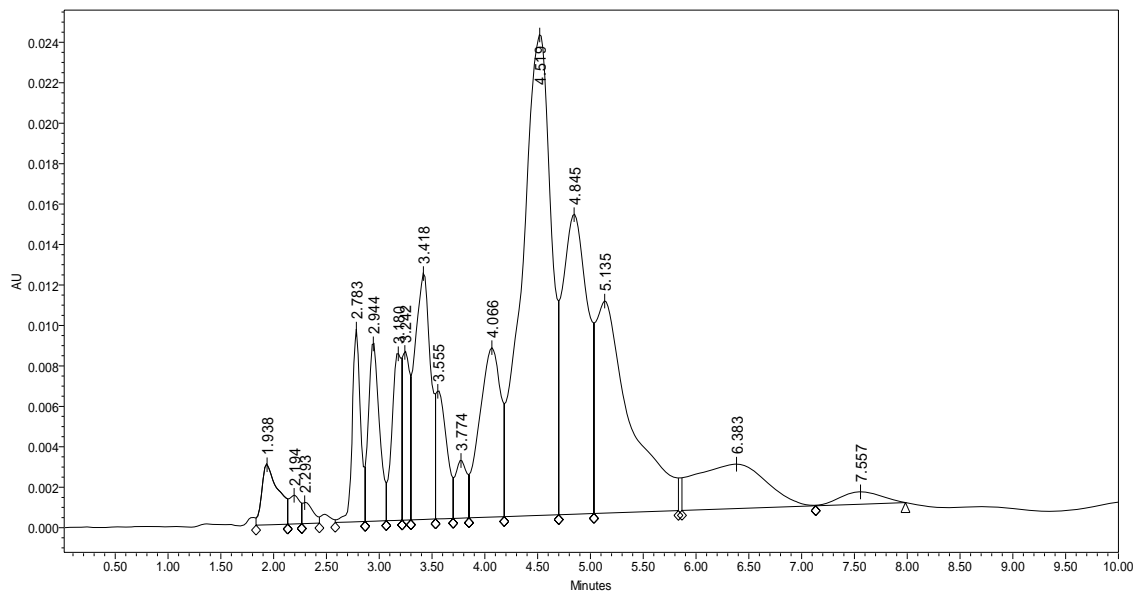


Figure 4.18: C. I. Reactive Blue 21 on Symmetry (C18) Using TBAHS and Gradient 1



Figures 4.19 and 4.20 show the blue dye on the Atlantis column with both ion-pair reagents.

Figure 4.19: C. I. Reactive Blue 21 on Atlantis (C18) Using TBAB and Gradient 1

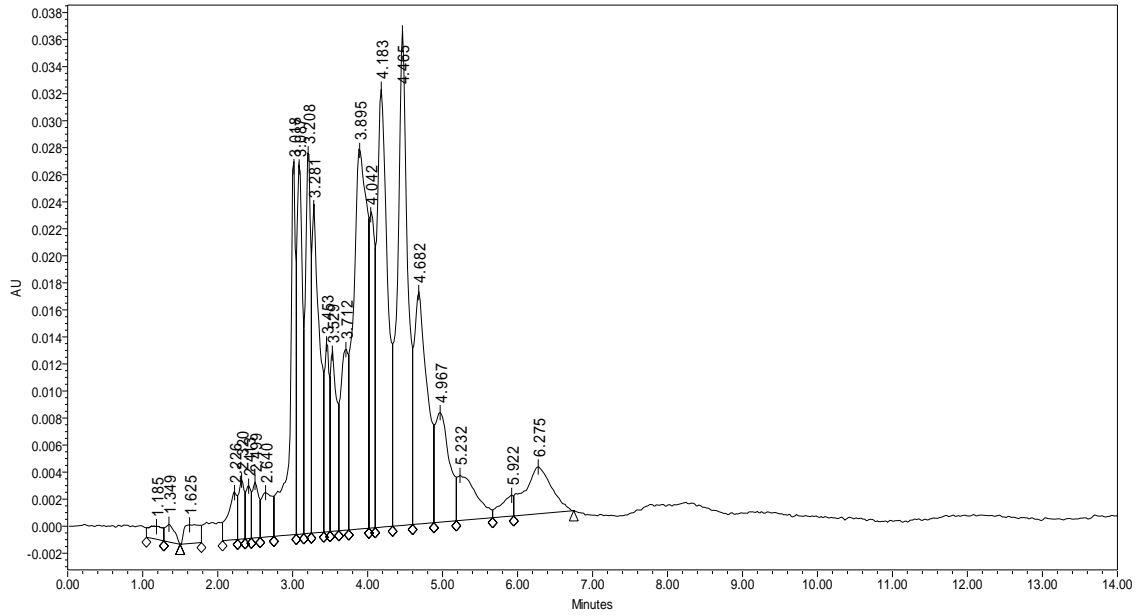
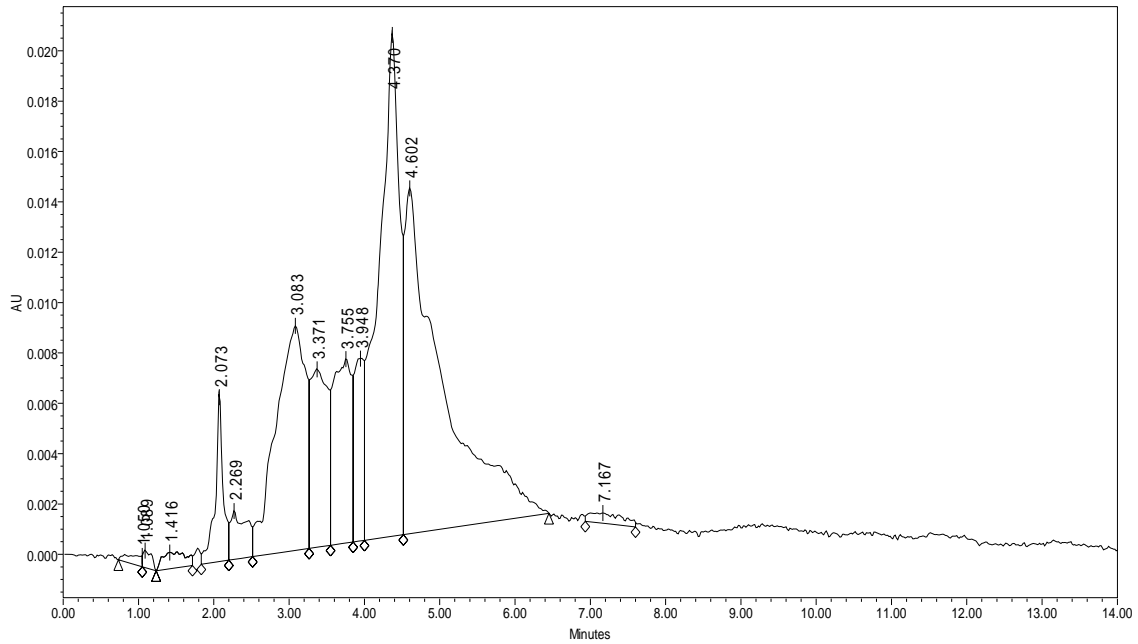


Figure 4.20: C. I. Reactive Blue 21 on Atlantis (C18) Using TBAHS and Gradient 1



By using the Atlantis column, improved separation of the components of Reactive Blue 21 is seen with the TBAB relative to TBAHS. The number of peaks separated greatly improved (Table 4.5), and the k' values (highest absorbance peak) are similar for both compounds.

Table 4.5 Resolved Peaks and k' Values for Reactive Blue 21 on Atlantis Column

Column	TBAB	TBAHS	Diff	k' for TBAB ($t_m = 4.5$)	k' for TBAHS ($t_m = 4.4$)
Atlantis	24	12	12	3.47	3.37

4.2 Stationary Phase and Gradient Experiments

The experiments conducted give very important information about the analysis of reactive dyes. The purpose of these experiments is to help determine an improved method for the analysis of reactive dyes by HPLC. This is done by examining the stationary phases (4.2.1) and the gradient system (4.2.2).

4.2.1 Stationary Phases

Different types of stationary phases were examined in order to determine which of the stationary phases yielded the best chromatograms for analysis of reactive dyes. Reversed phase HPLC stationary phases of interest studied include methyl (C_1), butyl (C_4), phenyl (C_6H_5), and octadecyl (C_{18}). Specific column characteristics can be seen in Table 3.2. Table 4.6 shows the gradients used in this experiment (Gradient 1 and 2). Columns with a length of 250mm were used with Gradient 2, while those with shorter lengths used with Gradient 1.

Table 4.6 Gradients 1 and 2

Flow (mL/min)	Gradient 1			Gradient 2		
	Time	A	B	Time	A	B
1	Initial	70	30	Initial	70	30
1	1.5	50	50	5.0	50	50
1	3.5	70	30	13.0	70	30

A= 30%B/H₂O + 0.05M NH₄H₂PO₄, B= 100% AN + 0.025 TBAB

The study of the effectiveness of these columns is described in Table 4.7 below where, based on the number of peaks resolved and overall peak shape, the columns were ranked as good (3), better (2), or best (1) for each dye analyzed on the column. An example of this qualitative method is shown for Reactive Blue 21 in Figures 4.21, 4.22, and 4.23, which are chromatograms of the dye on the Novapak, Zorbax Reliance, and the Atlantis columns respectively.

Table 4.7 Column Comparision for Reactive Dyes

C.I. Name	Adsorbo-sphere	Alltima	Atlantis	Exsil Butyl	Exsil ODS	Exsil TMS	Nova-Pak	Sym-metry	Zorbax Reliance
Red 43	N/A	1.5	2	N/A	2	N/A	3	3	1
Red 180	N/A	2	2	2	1	N/A	3	3	2
Red 231	3	2	1	N/A	3	N/A	3	3	3
Yellow 3	3	2	1	3	3	N/A	3	3	2
Yellow 135	3	2	1	2	2	N/A	3	3	3
Yellow 168	3	3	2	2	1	N/A	3	2	3
Blue 21	N/A	2	1	2	2	N/A	3	3	2
Blue 52	N/A	3	1	N/A	2	N/A	3	2	2
Blue 238	N/A	2	1	N/A	3	N/A	3	2	2
Column SP	C18	C6H5	C18	C4	C18	C1	C18	C18	C6H5

Figure 4.21: C. I. Reactive Blue 21 on Novapak (C18) Using TBAB (3-Good) with Gradient 1

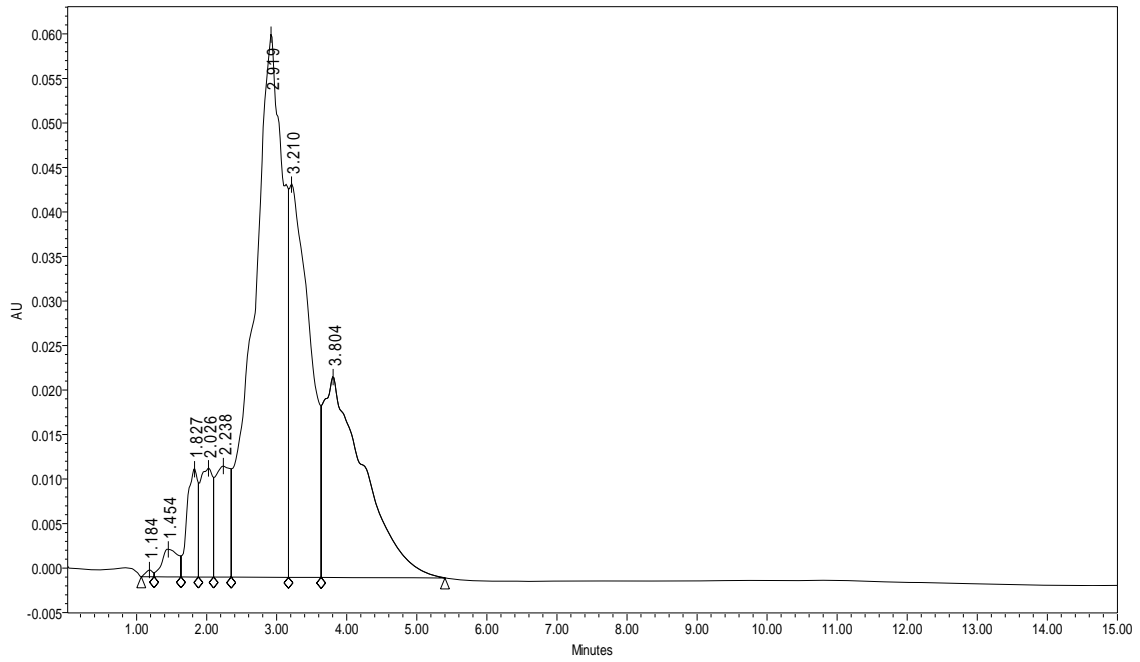


Figure 4.22: C. I. Reactive Blue 21 on Zorbax Reliance (C18) (2-Better) with Gradient 1

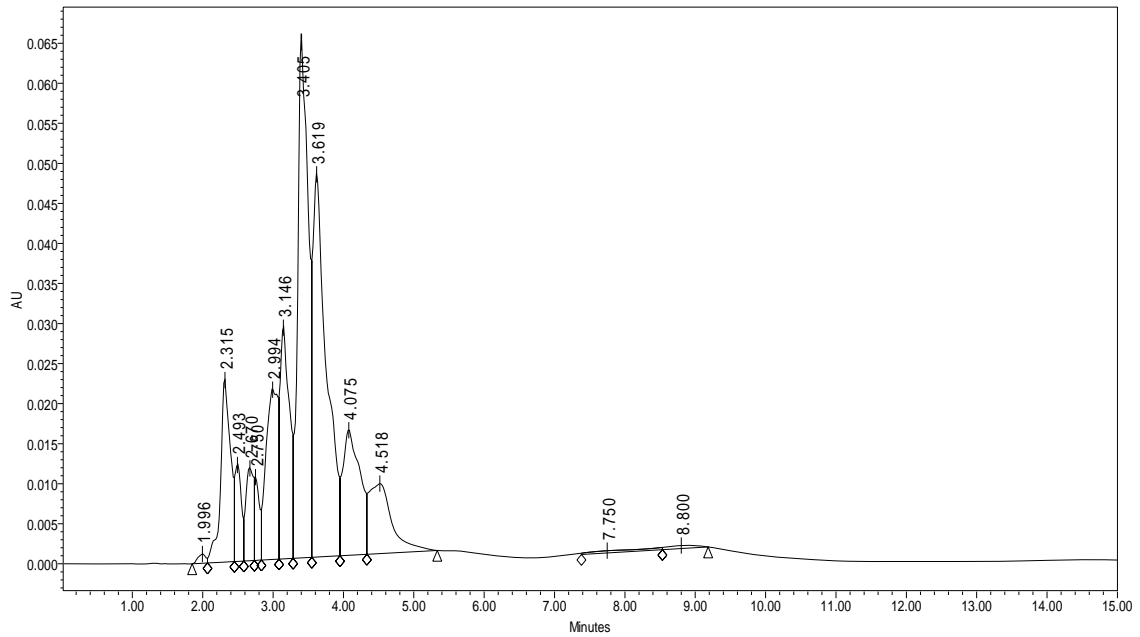


Figure 4.23: C. I. Reactive Blue 21 on Atlantis (C18) (1-Best) with Gradient 1

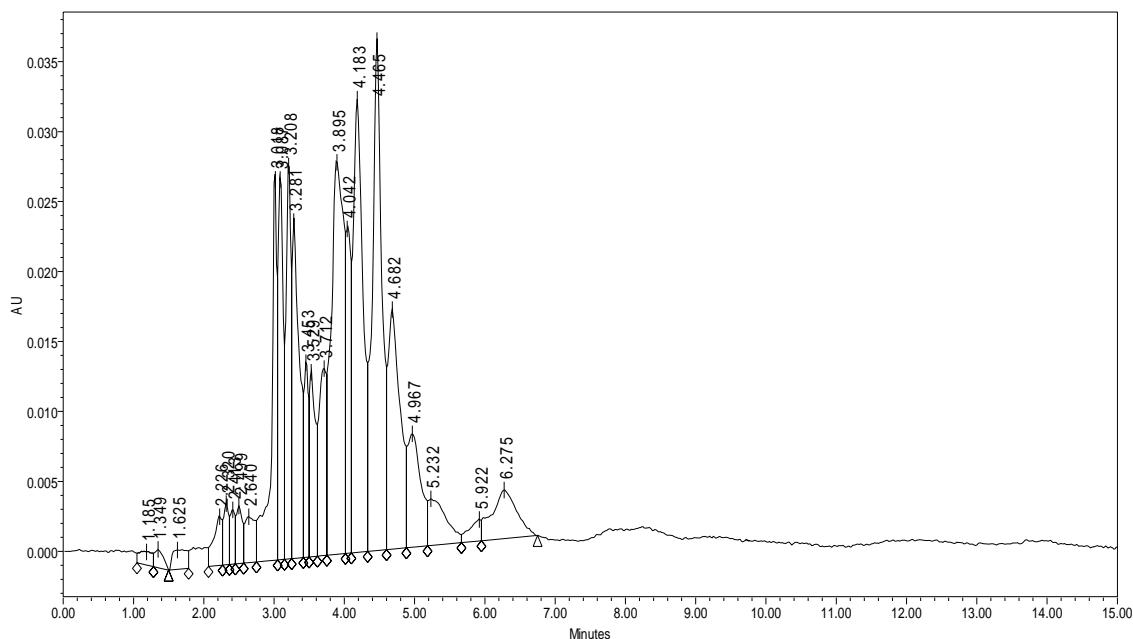


Table 4.7 summarizes the information that was obtained by a series of analyses. The C₁ (TMS) and C₄ (Butyl) stationary phases do not provide enough carbon loading for sufficient analyte interaction with the column. Because of this, efficient separation could not be achieved for many of the reactive dyes using these columns. Both of the C₆H₅ columns (Alltima and Zorbax Reliance) gave good results that were very similar in spite of the fact that the column characteristics are extremely different. The Alltima column was long (250mm) and had larger particles (5- μ m), while the Zorbax Reliance was very short (40mm) with smaller particles (3- μ m). For the C₁₈ columns, the best performer was the Atlantis column, followed the Exsil ODS.

Based on these results, the column characteristic that is key for the analysis of these reactive dyes is the chemistry of the stationary phase. Though all C₁₈ columns have the same

basic ligand group, some column chemistries vary, and new technologies have allowed companies to tailor these basic ligand groups for more specific analytical purposes. For example, the Atlantis C₁₈ is designed to improve the retention of polar compounds on the stationary phase. The ligand is attached to the support phase (silica gel) by two Si-O-Si bonds, which leads to increased column life. The ligand loading is also lower than other C₁₈ phases, leading to higher residual SiOH groups to interact with polar analytes [10].

4.2.2 Reactive Blue 21 Gradient Adjustments

The reactive dyes utilized in this study were separated very well using either Gradient 1 or Gradient 2 found in Table 4.6, with the exception of Reactive Blue 21. Though the separation of the dye was greatly improved with the use of the Atlantis column (Figure 4.19), additional factors were examined in order to further enhance this separation. Factors including gradient elution, gradient curve, and time were all examined.

4.2.2.1 Gradient Elution

Gradient elution for Reactive Blue 21 was conducted on the Atlantis column using Gradient 1 (Table 4.6) as the basis. The solvent percentages were changed for the initial and final times of the gradient, and were the same for both. An example of this is shown in Table 4.8 below for the 80_20 gradient and the 65_35 gradient. Figures 4.24, 4.25, 4.26, 4.27, and 4.28 are the chromatograms of Reactive Blue 21 with the gradient elution beginning and ending at 90/10, 80/20, 70/30, and 60/40 respectively, and Table 4.9 shows the number of peaks separated with each change of the gradient.

Table 4.8: C. I. Reactive Blue 21 Atlantis (C18) Gradient Elution Adjustment Example

	80/20 Gradient			65/30 Gradient		
Flow (mL/min)	Time	A	B	Time	A	B
1	Initial	80	20	Initial	65	35
1	1.5	50	50	1.5	50	50
1	3.5	80	20	3.5	65	35

A= 30%B/H₂O + 0.05M NH₄H₂PO₄, B= 100% AN + 0.025 TBAB

Figure 4.24: C. I. Reactive Blue 21 Atlantis (C18) with 90_10 Gradient

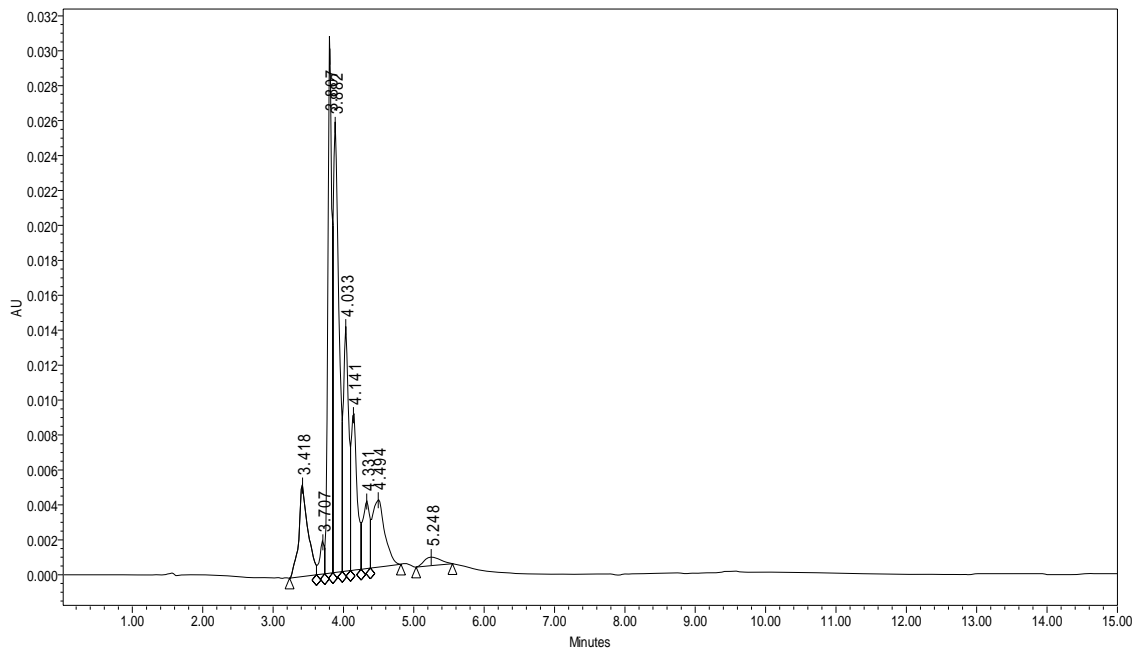


Figure 4.25: C. I. Reactive Blue 21 Atlantis (C18) with 80_20 Gradient

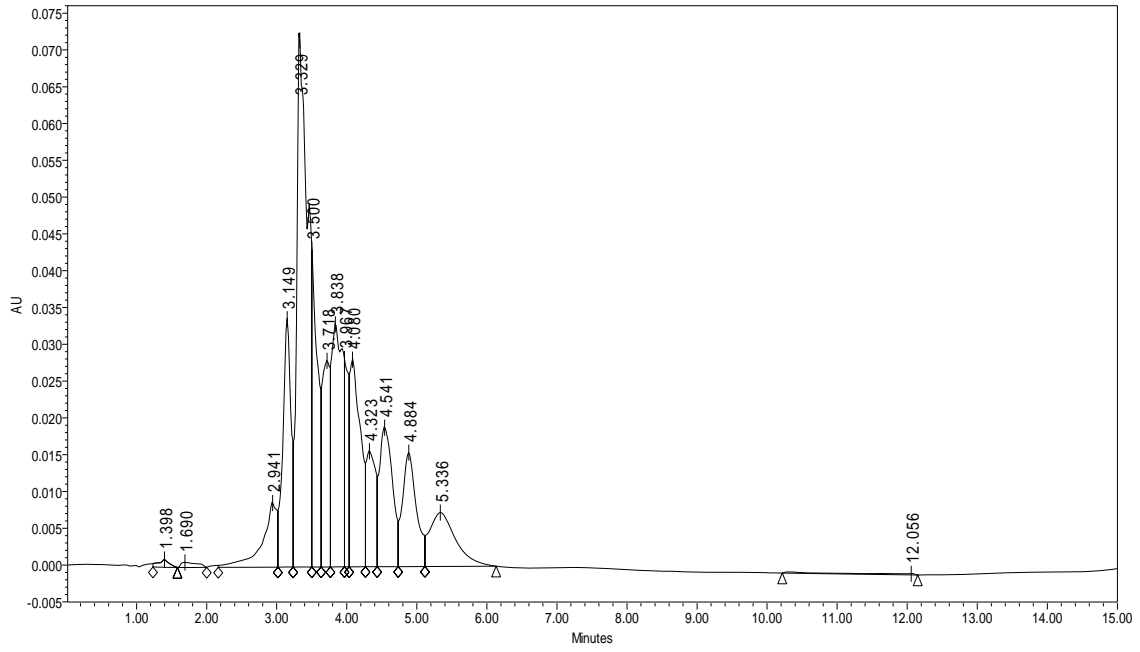


Figure 4.26: C. I. Reactive Blue 21 Atlantis (C18) with 70_30 Gradient

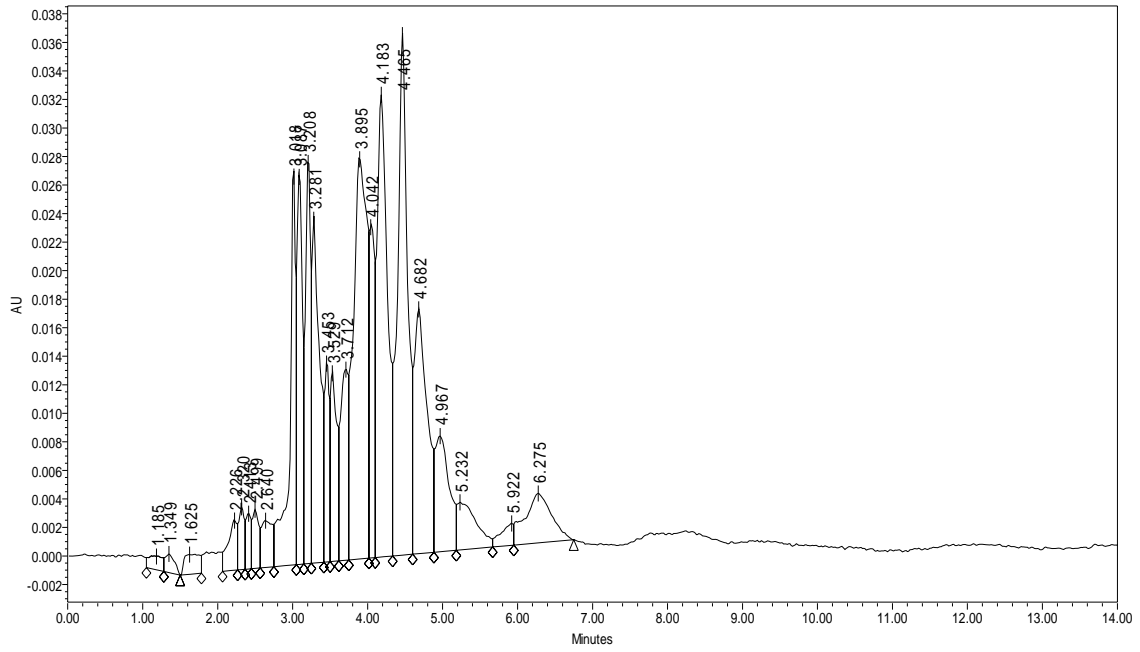


Figure 4.27: C. I. Reactive Blue 21 Atlantis (C18) with 65_35 Gradient

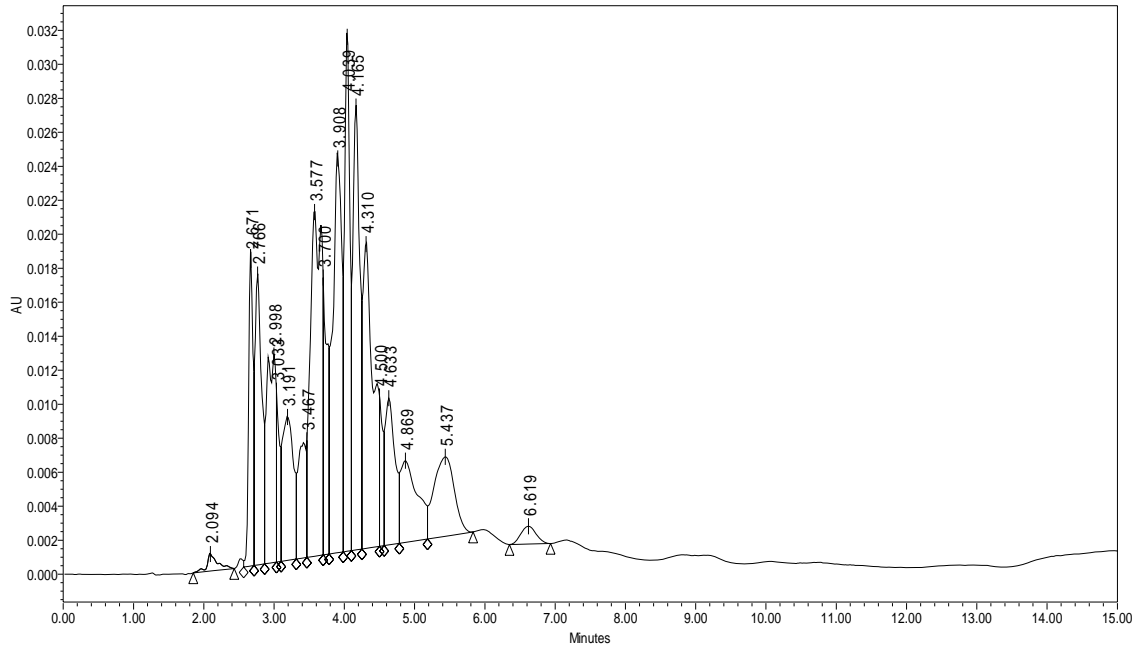


Figure 4.28: C. I. Reactive Blue 21 Atlantis (C18) with 60_40 Gradient

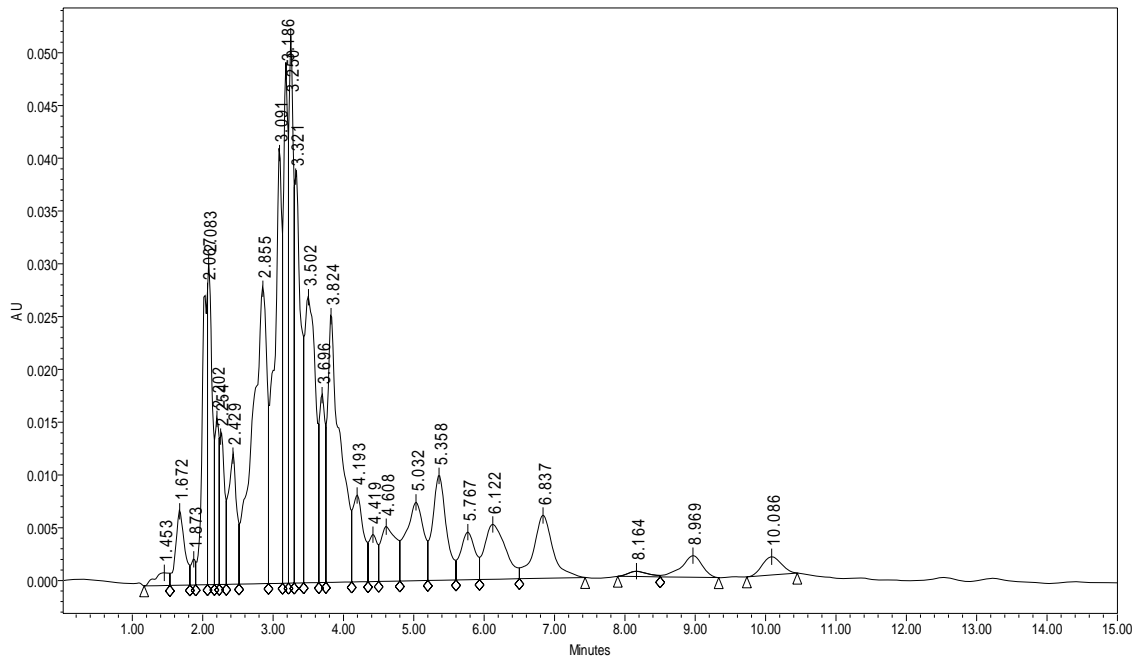


Table 4.9 Number of Peaks Resolved for C. I. Reactive Blue 21 Gradient Changes

%A/%B	# of Peaks
90/10	9
80/20	15
70/30	24
65/35	22
60/40	27

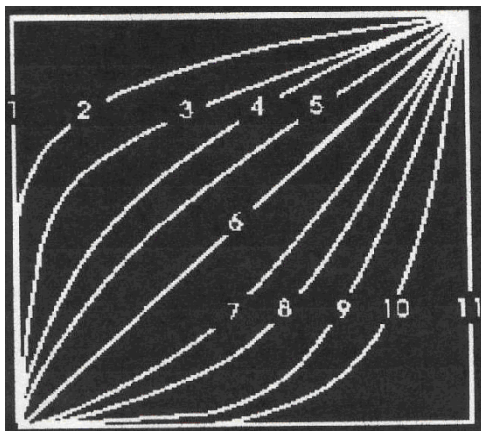
A= 30%B/H₂O + 0.05M NH₄H₂PO₄ , B= 100% AN + 0.025 TBAB

By examining the chromatograms and Table 4.8, it can be seen that the greatest number of peaks is resolved using the 60/40 gradient. This is a somewhat unusual trend, since when you slightly increase the organic phase, the number of peaks that separate increased relative to the 70/30 gradient, decreases, and then increased again for the 60/40 gradient. Also, the 60/40 gradient spreads out the peaks by approximately 2x the time that it takes for the 70/30 gradient. Since that gradient spreads out the peaks slightly more, it takes slightly more time for the column to regenerate, thus prolonging the actual run for the 60/40 gradient, in comparison to the other gradients in the study.

4.2.2.2 Gradient Curve

Gradient curve can assist in either speeding up or slowing down the rate of change of gradient formation. The profile of the gradient curves in the Waters Alliance System can be seen in Figure 4.29, where the gradient curve 6 is a linear gradient separation, where mobile phase A and B change at the same rate to reach the next gradient step. Gradient curves 2-5 increased the rate of change as it approaches curve 1, while curves 7-10 slow it down as it approaches curve 11.

Figure 4.29: Profile of Gradient Curves



Figures 4.30, 4.31, 4.32, 4.33, 4.34, 4.35, and 4.36 are the chromatograms of Reactive Blue 21 on the Atlantis column using Gradient 1, with the gradient curve changed to 2, 4, 6 (linear and standard), 7, 8, 9, and 10 respectively. A summary of the number of peaks observed for these curve changes is seen in Table 4.10.

Figure 4.30: C. I. Reactive Blue 21 Atlantis (C18) with Gradient Curve 2 and Gradient 1

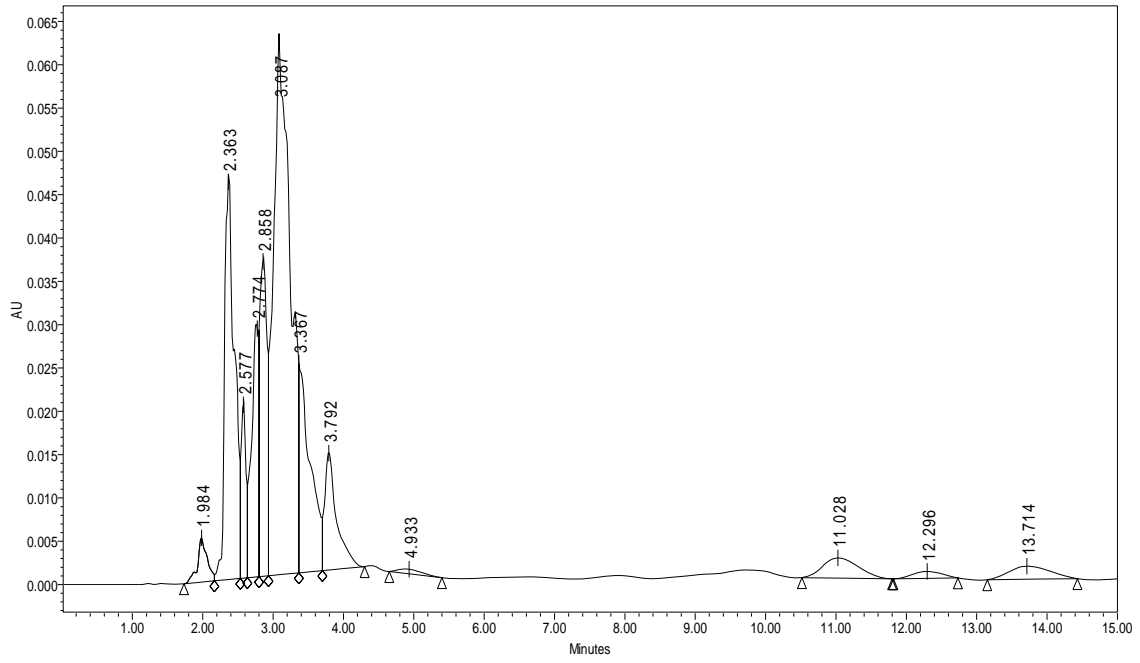


Figure 4.31: C. I. Reactive Blue 21 Atlantis (C18) with Gradient Curve 4 and Gradient 1

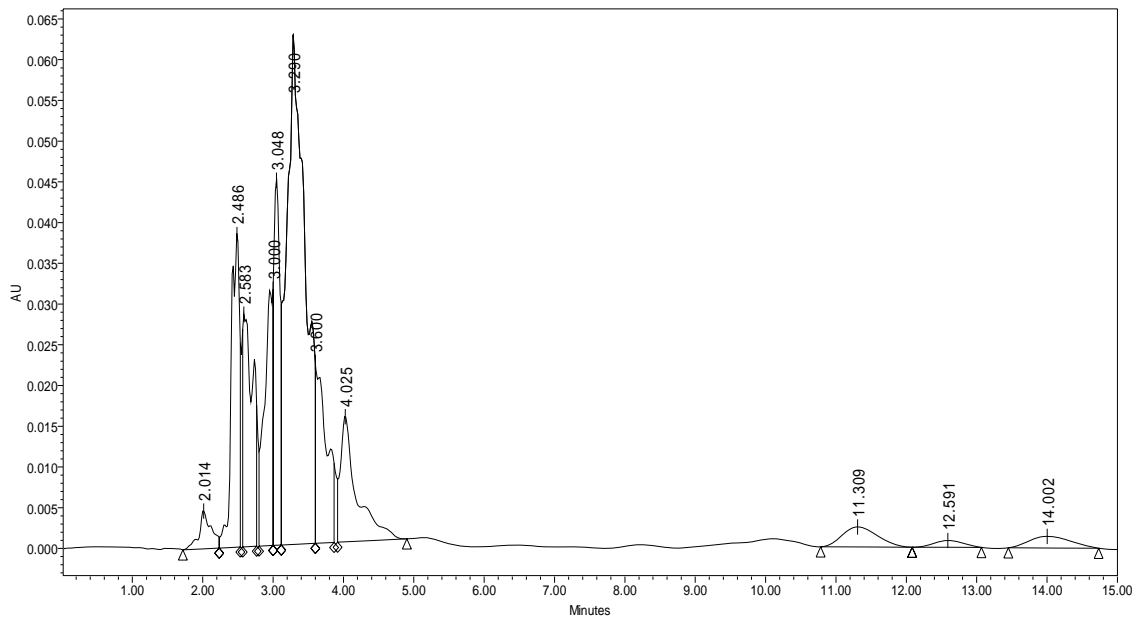


Figure 4.32: C. I. Reactive Blue 21 Atlantis (C18) with Gradient Curve 6 and Gradient 1

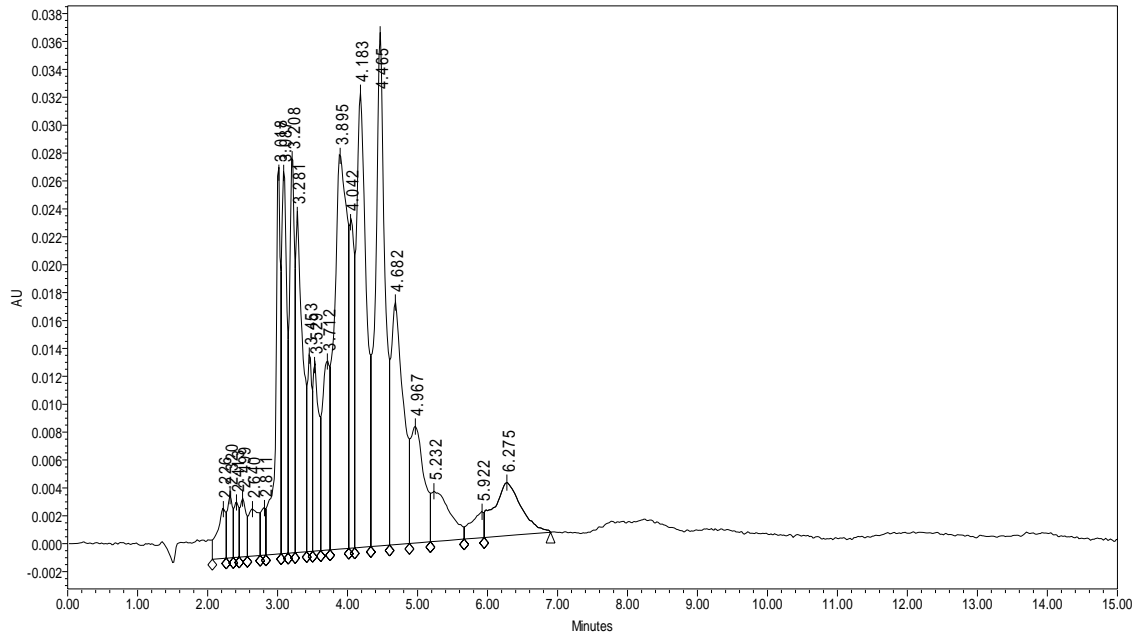


Figure 4.33: C. I. Reactive Blue 21 Atlantis (C18) with Gradient Curve 7 and Gradient 1

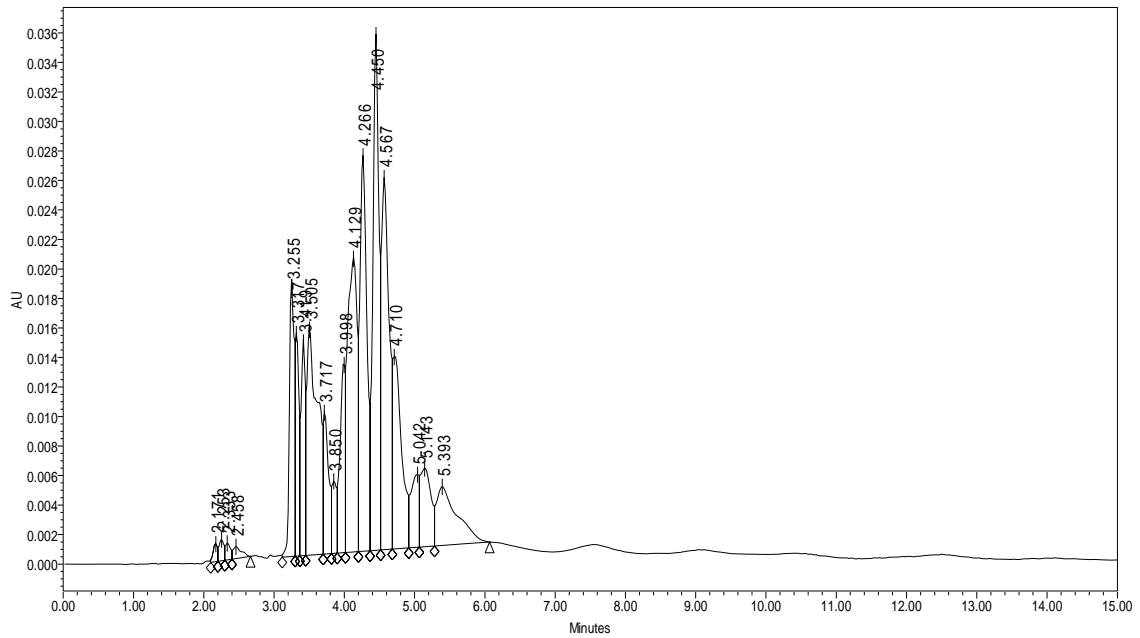


Figure 4.34: C. I. Reactive Blue 21 Atlantis (C18) with Gradient Curve 8 and Gradient 1

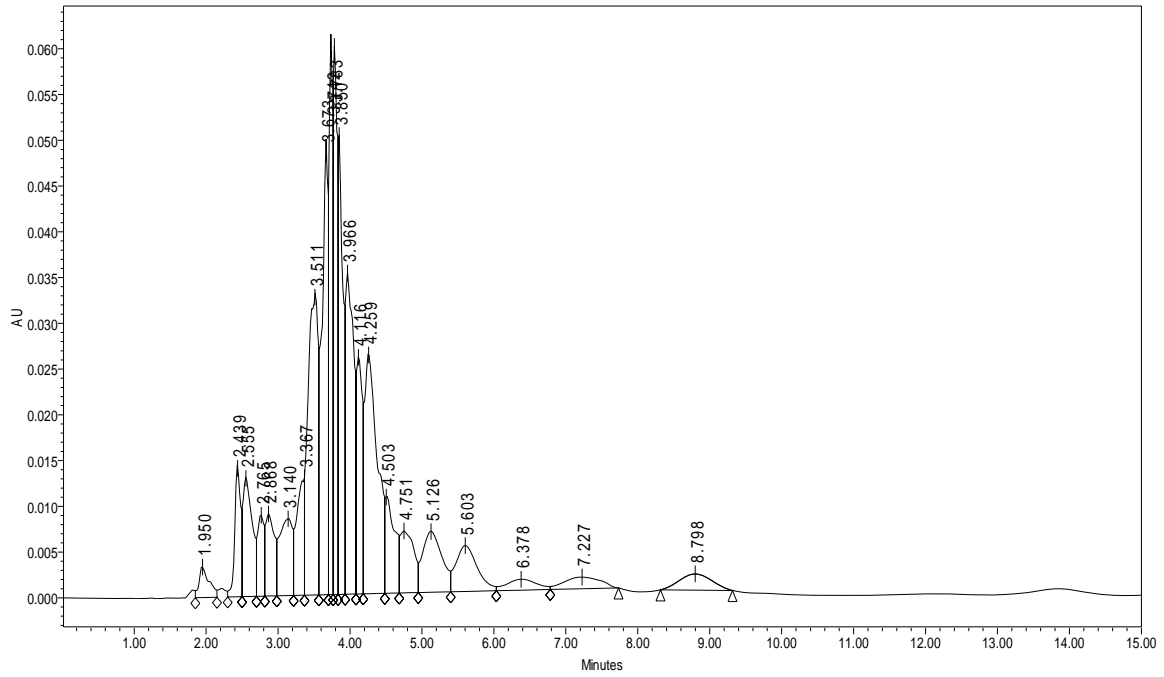


Figure 4.35: C. I. Reactive Blue 21 Atlantis (C18) with Gradient Curve 9 and Gradient 1

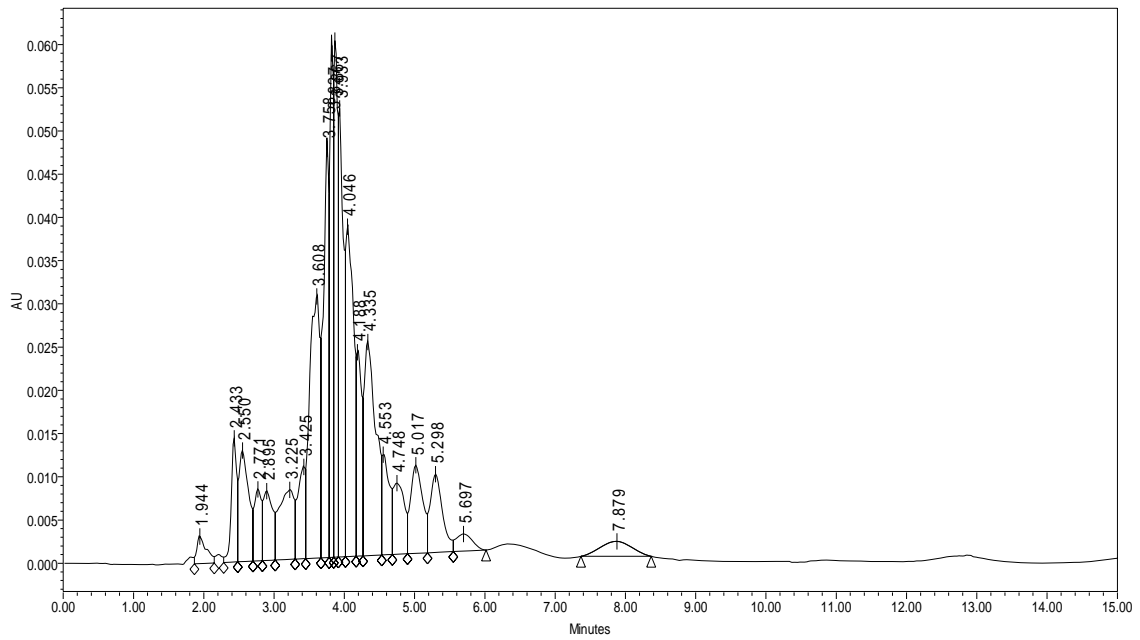


Figure 4.36: C. I. Reactive Blue 21 Atlantis (C18) with Gradient Curve 10 and Gradient 1

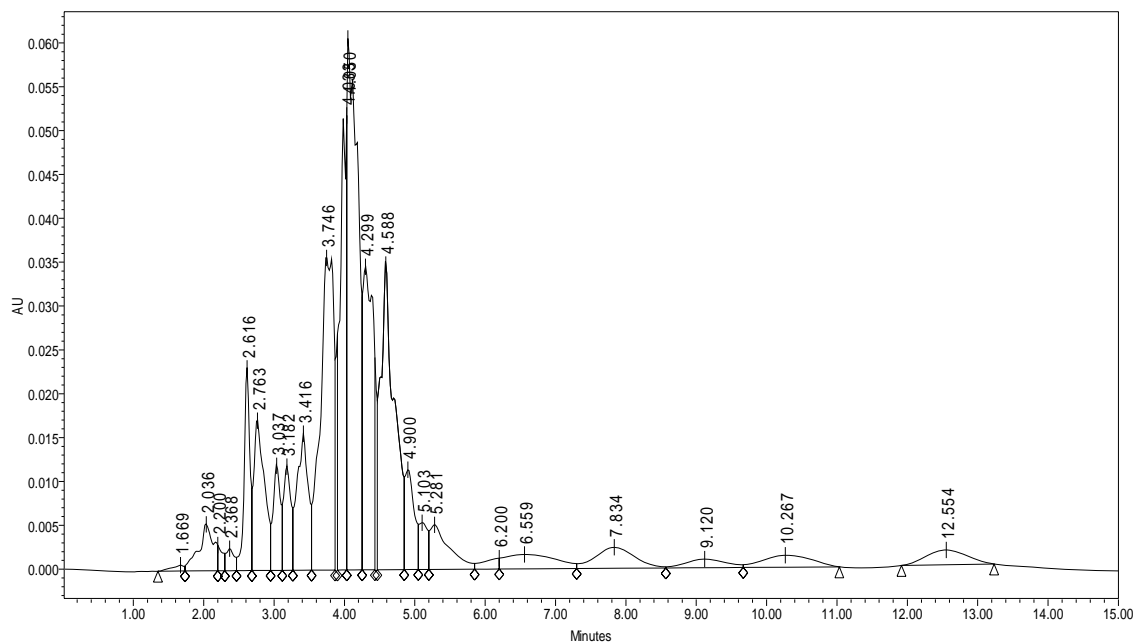


Table 4.10 Number of Peaks Resolved for C.I. Reactive Blue 21 Gradient Curve

Curve	# of Peaks
2	12
4	12
6	24
7	27
8	22
9	21
10	23

The number of peaks separated is best with the gradient curve 7, which slightly slows the initial gradient change.

4.2.2.3 Gradient Time

The gradient used in the experiment can be further manipulated by adjusting the gradient time intervals. Gradients 1 and 2 both gave satisfactory results for most reactive

dyes. However, Reactive Blue 21 is a more complex dye than most, so it would be of interest to see if allowing this dye more time to interact with the stationary phase would improve separation. This was achieved by decreasing the slope of the linear gradient curve. An example of this is shown in Table 4.11 below for the 4_6 and the 12_14 gradients.

Table 4.11 Linear Gradient Time Adjustment Example

Flow (mL/min)	4_6 Gradient			12_14 Gradient		
	Time	A	B	Time	A	B
1	Initial	70	30	Initial	70	30
1	4.0	50	50	12.0	50	50
1	6.0	70	30	14.0	70	30

A= 30%B/H₂O + 0.05M NH₄H₂PO₄ , B= 100% AN + 0.025 TBAB

Figures 4.37, 4.38, 4.39, 4.40, 4.41, 4.42, 4.43, 4.44, 4.45, 4.46, 4.47, 4.48, 4.49, and 4.50 are the chromatograms of Reactive Blue 21 on the Atlantis column using the gradient time changes. Table 4.12 summarizes these results.

Figure 4.37: C. I. Reactive Blue 21 Atlantis (C18) with 1.5_3.5 Gradient Time

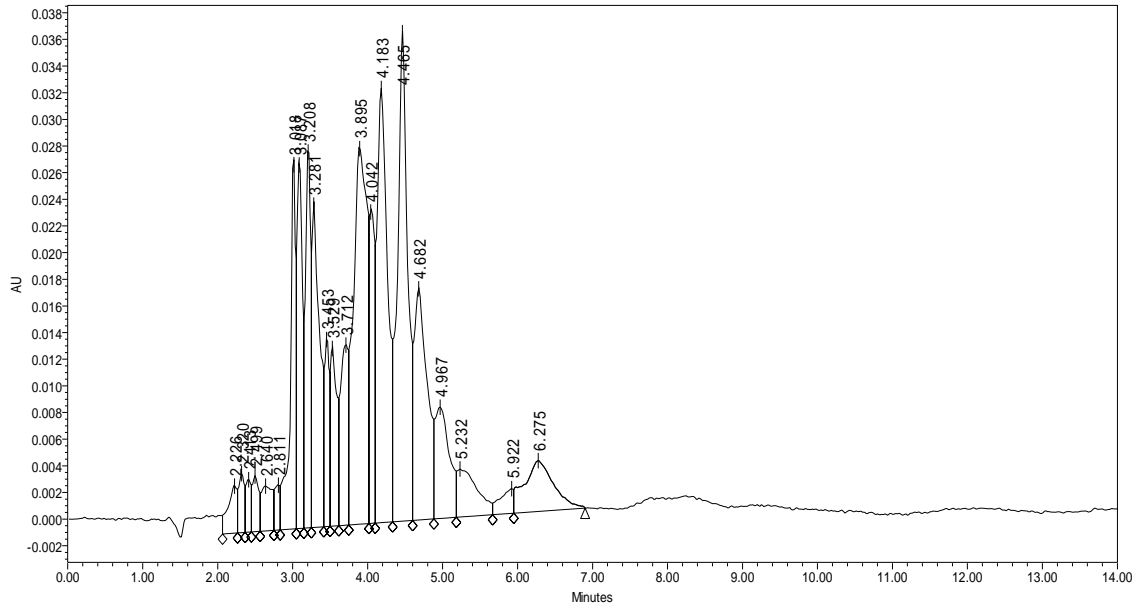


Figure 4.38: C. I. Reactive Blue 21 Atlantis (C18) with 2_4 Gradient Time

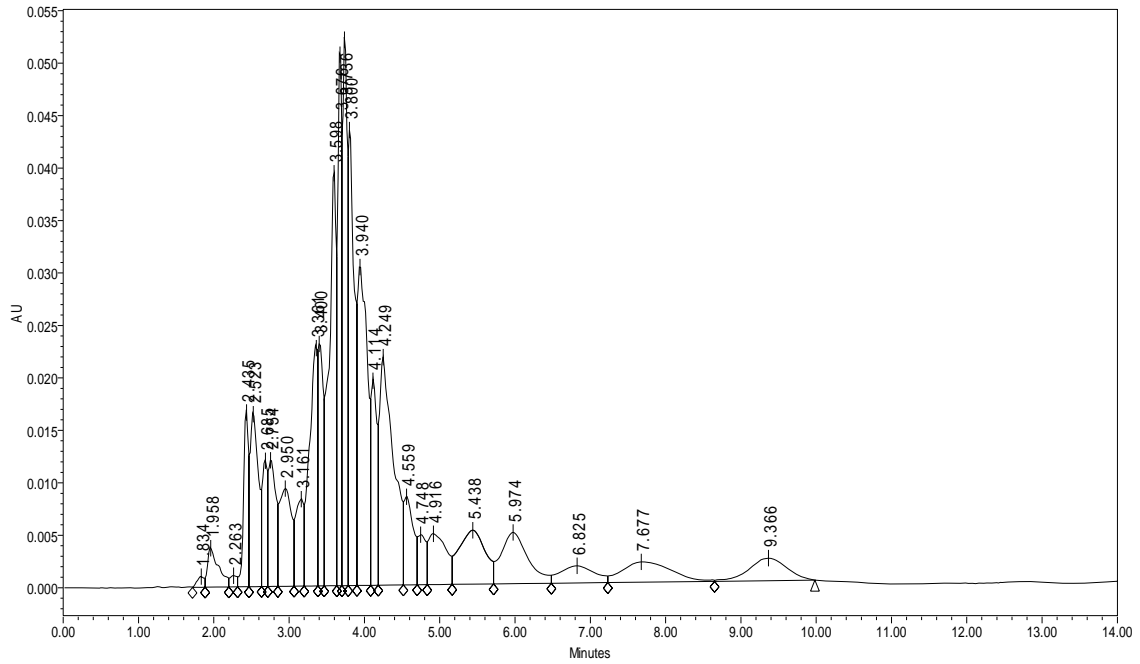


Figure 4.39: C. I. Reactive Blue 21 Atlantis (C18) with 3_5 Gradient Time

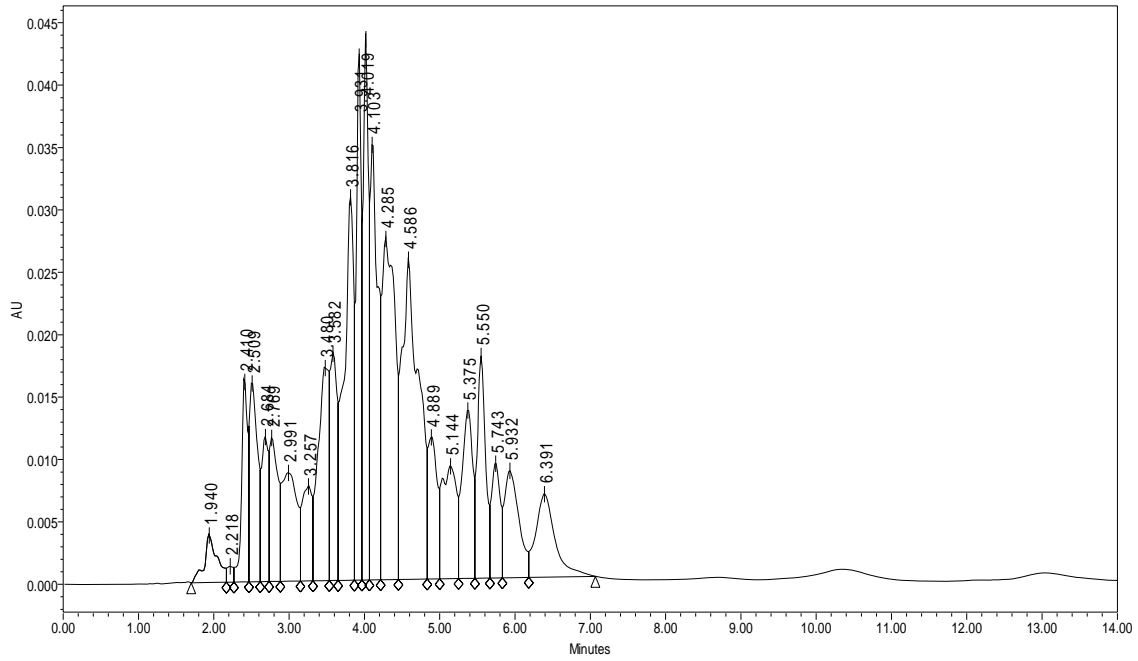


Figure 4.40: C. I. Reactive Blue 21 Atlantis (C18) with 4_6 Gradient Time

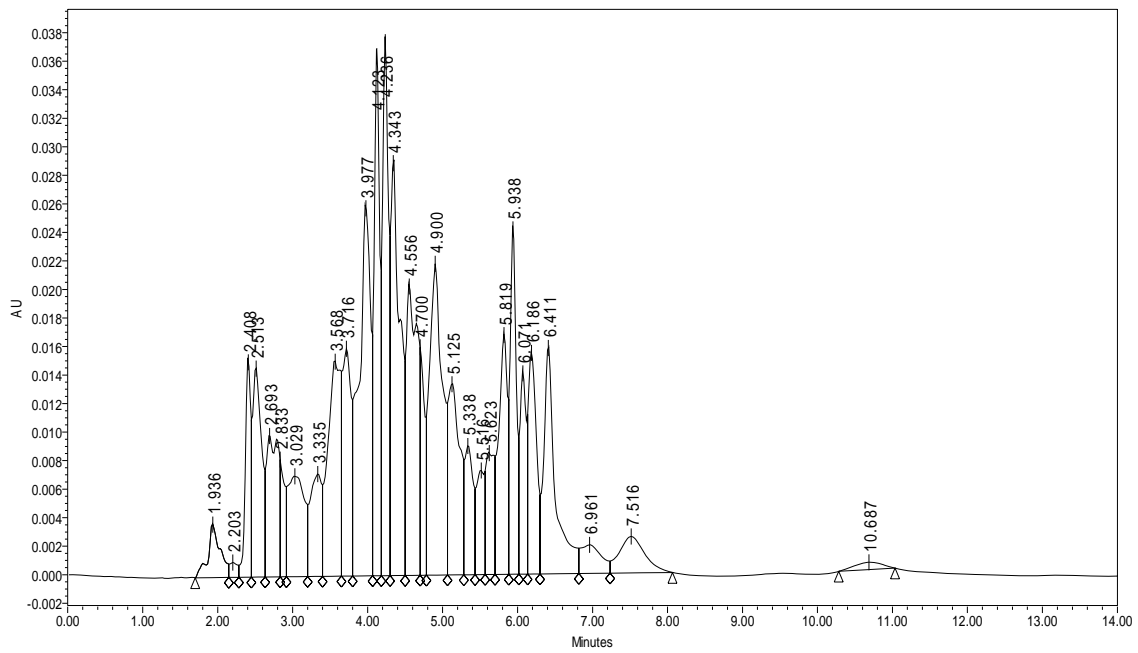


Figure 4.41: C. I. Reactive Blue 21 Atlantis (C18) with 5_7 Gradient Time

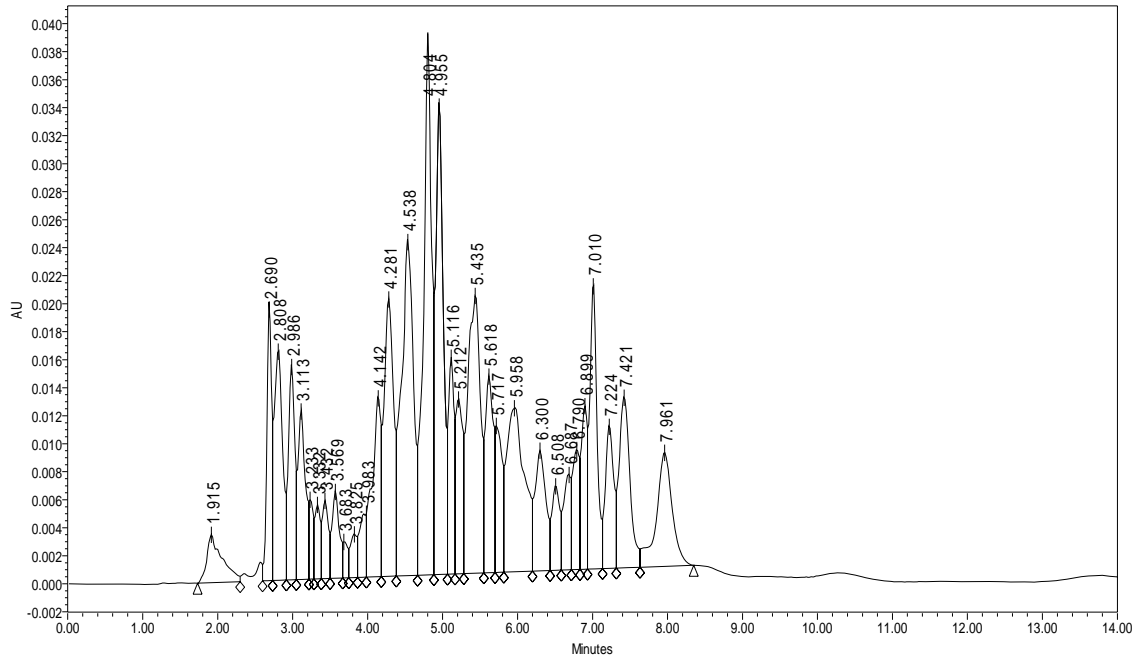


Figure 4.42: C. I. Reactive Blue 21 Atlantis (C18) with 6_8 Gradient Time

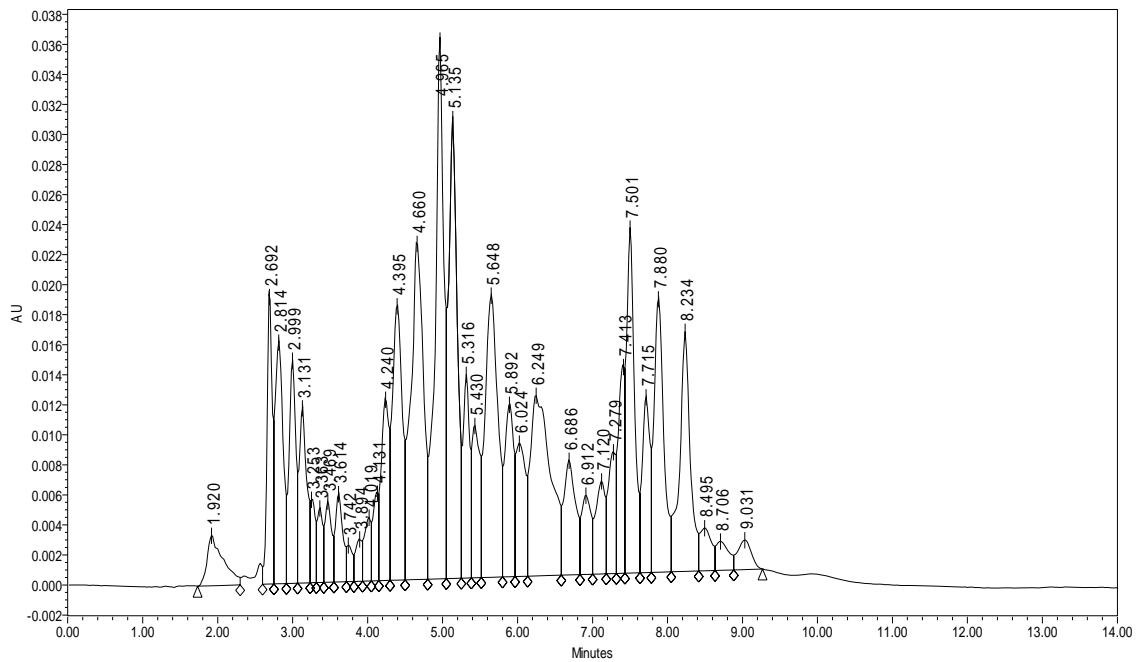


Figure 4.43: C. I. Reactive Blue 21 Atlantis (C18) with 7_9 Gradient Time

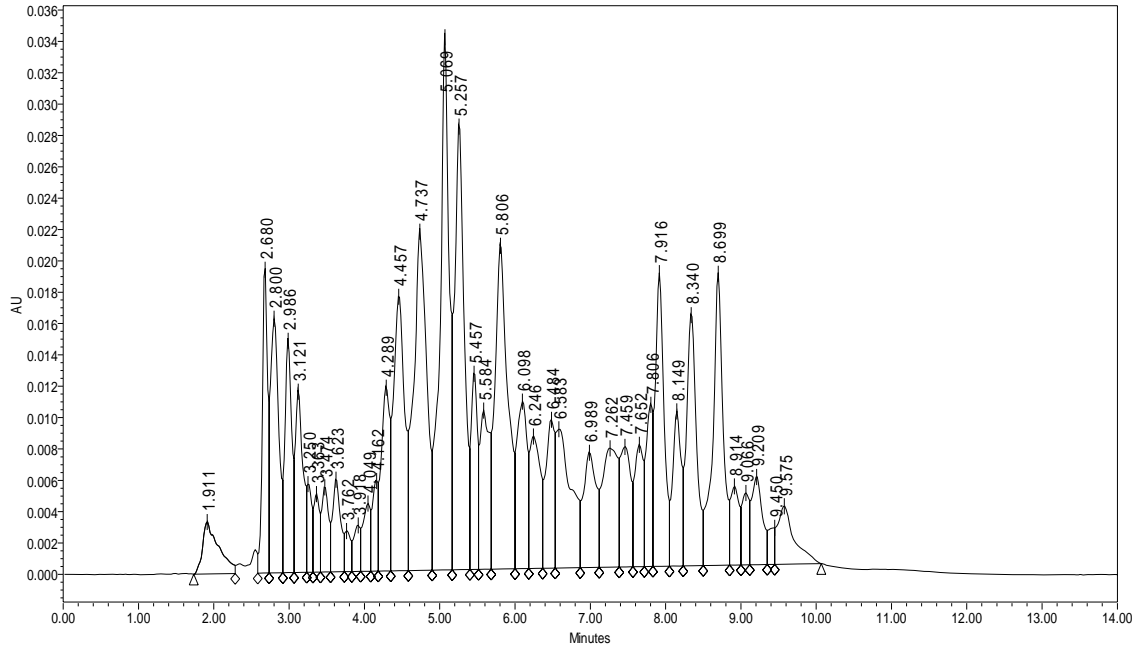


Figure 4.44: C. I. Reactive Blue 21 Atlantis (C18) with 8_10 Gradient Time

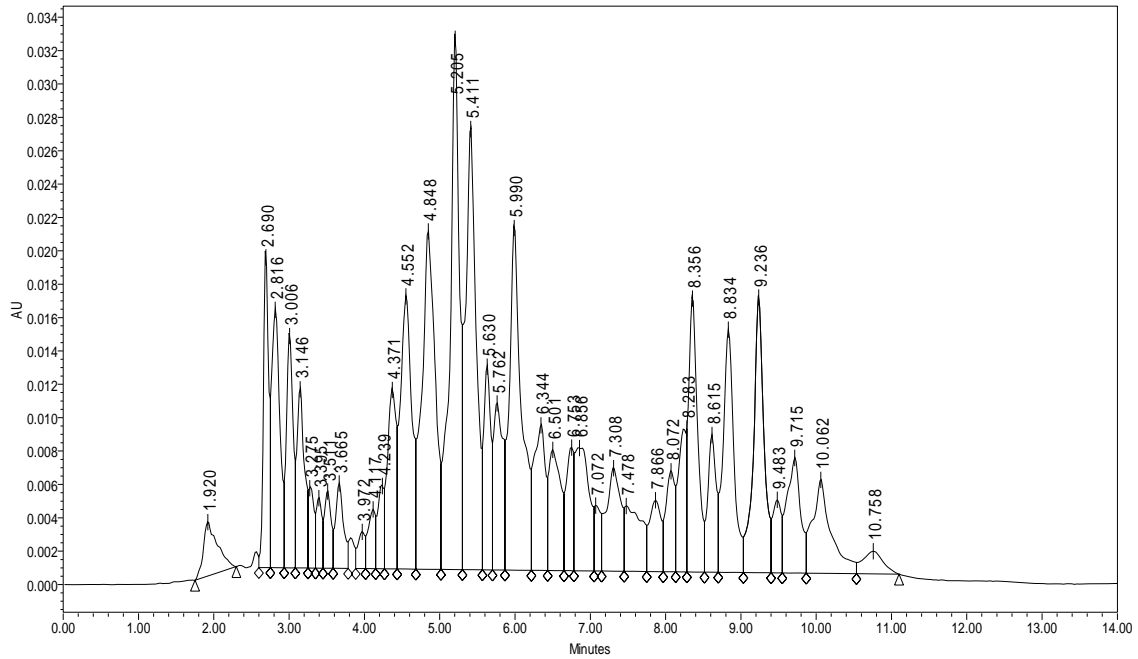


Figure 4.45: C. I. Reactive Blue 21 Atlantis (C18) with 9_11 Gradient Time

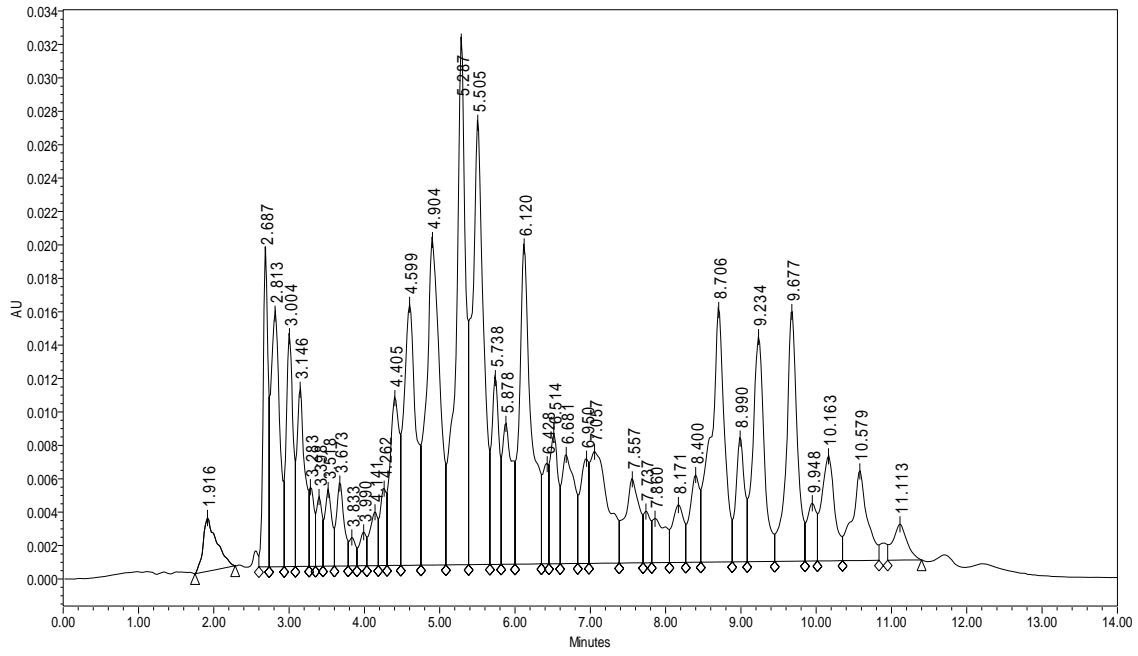


Figure 4.46: C. I. Reactive Blue 21 Atlantis (C18) with 10_12 Gradient Time

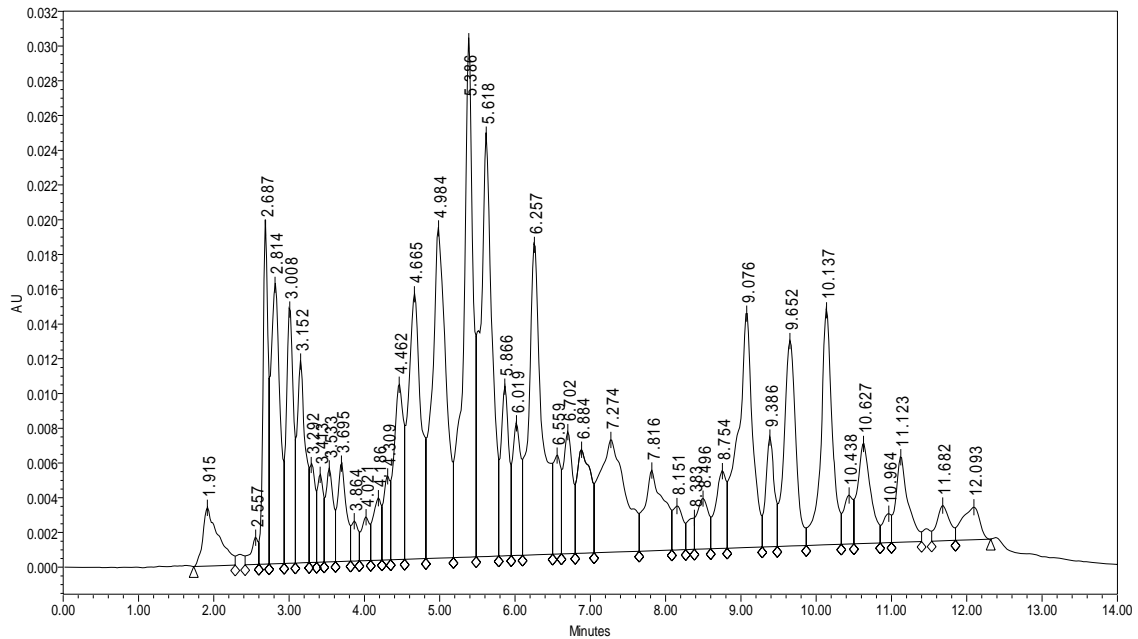


Figure 4.47: C. I. Reactive Blue 21 Atlantis (C18) with 11_13 Gradient Time

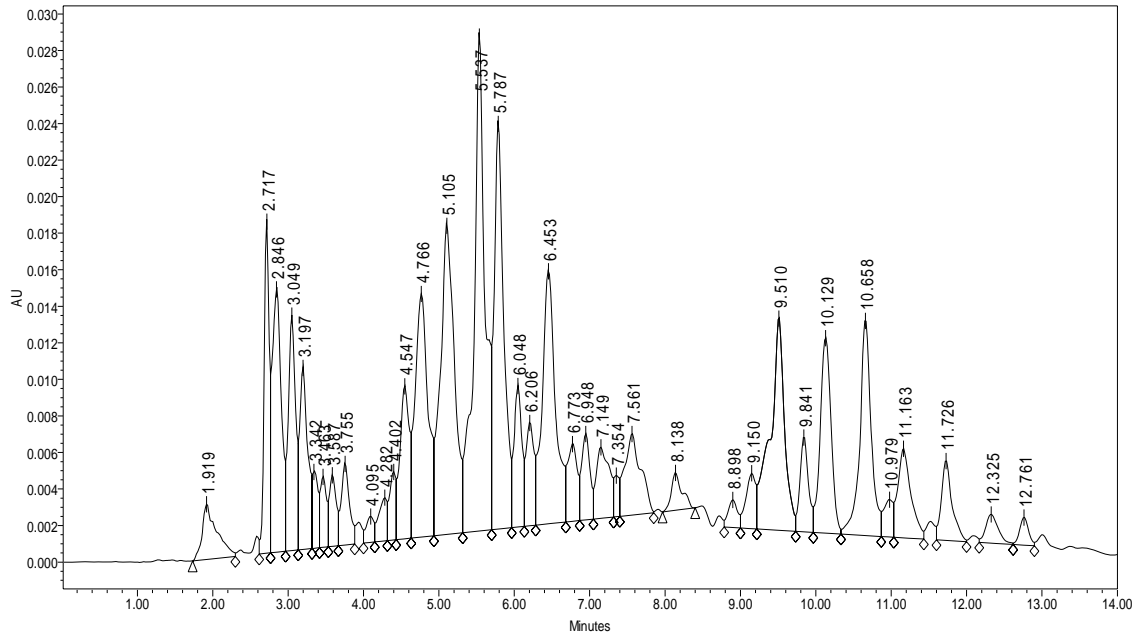


Figure 4.48: C. I. Reactive Blue 21 Atlantis (C18) with 12_14 Gradient Time

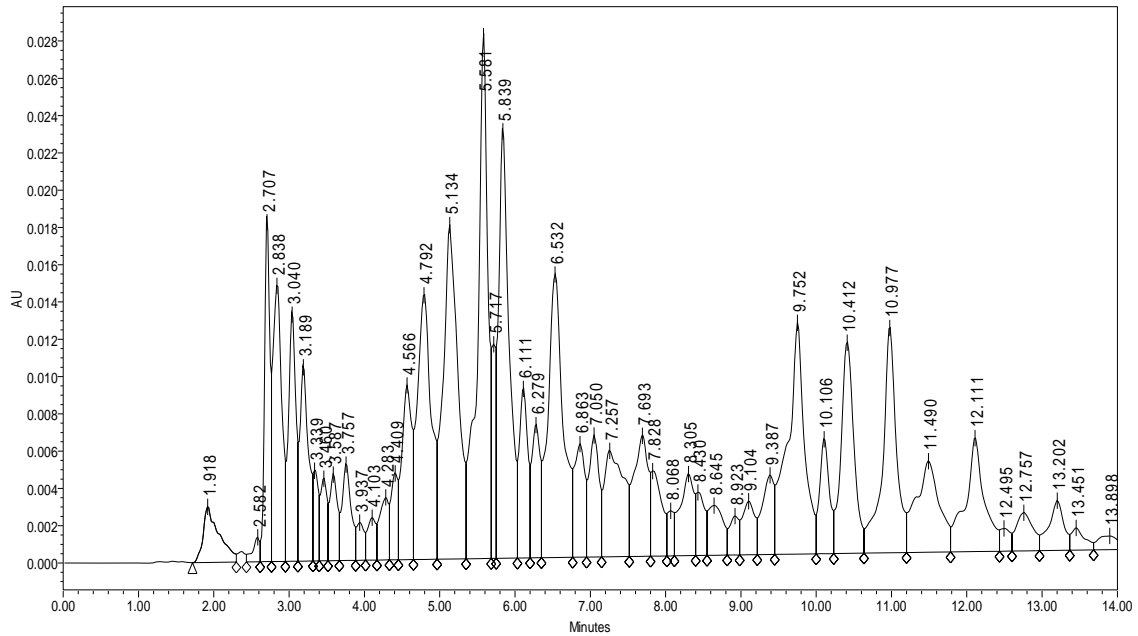


Figure 4.49: C. I. Reactive Blue 21 Atlantis (C18) with 13_15 Gradient Time

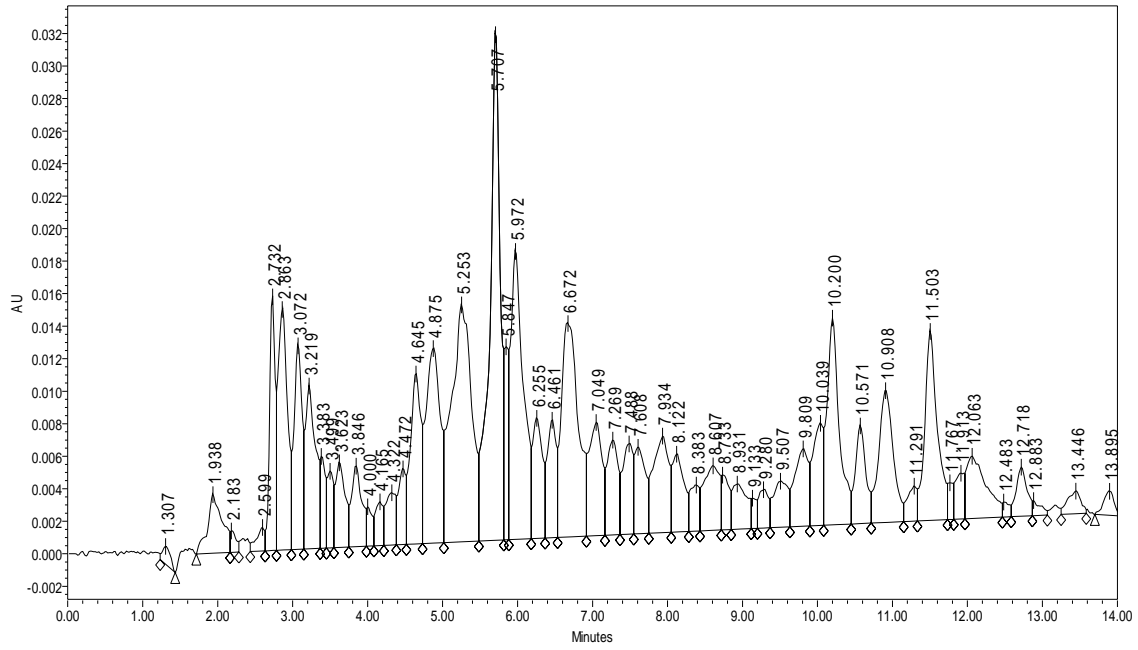


Figure 4.50: C. I. Reactive Blue 21 Atlantis (C18) with 14_16 Gradient Time

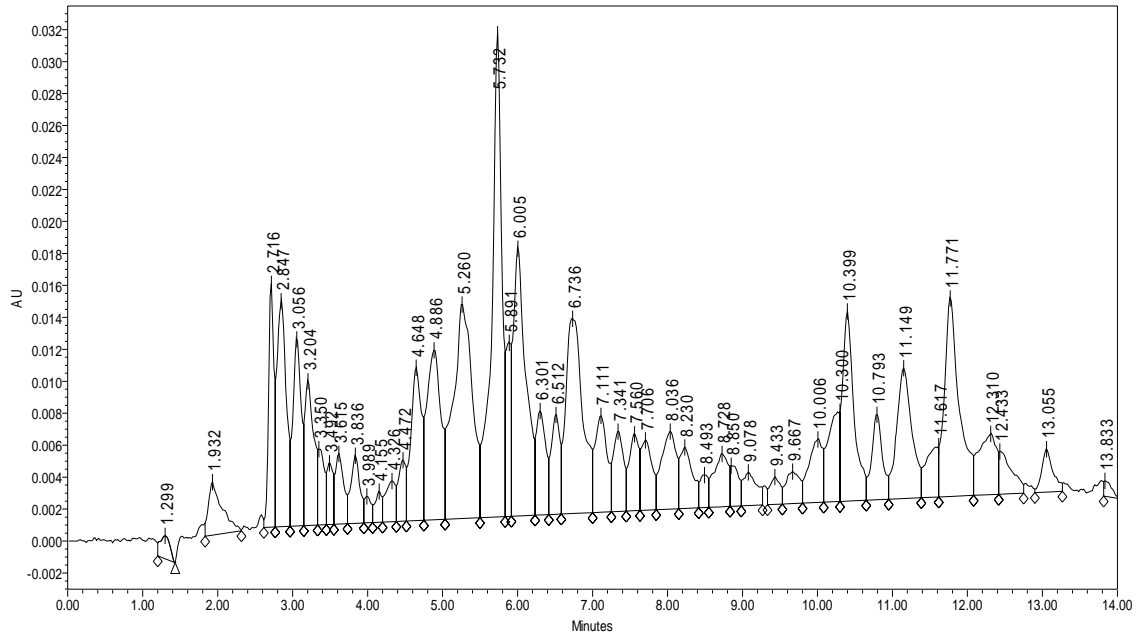


Table 4.12 Number of Resolved Peaks for C. I. Reactive Blue 21 Time

Time	# of Peaks
1.5 3.5	24
2 4	26
3 5	23
4 6	29
5 7	32
6 8	36
7 9	39
8 10	38
9 11	39
10 12	41
11 13	37
12 14	46
13 15	54
14 16	49

As the slope of the gradient decreases, the number of peaks generally increases. By increasing the gradient time, run time is also increased, so mobile phase used and experiment time is increased, at least by 2x compared to Gradient 1. Considering the peak shape, number of separated peaks, and column regeneration, the optimal method considering time, is the 10_12 gradient for Reactive Blue 21.

4.2.2.4 Gradient Optimization

Since adjusting the gradient time yielded the most peaks thus far for Reactive Blue 21, it was of interest to examine whether the other gradient factors could further improve this gradient. Table 4.13 shows the number of separated peaks for the figures listed below. Figure 4.51 is a chromatogram of Reactive Blue 21 on the Atlantis column with 10_12 gradient and gradient curve 7, Figure 4.52 shows the effect of the 10_12 gradient with 60/40 gradient elution on the chromatogram, and Figure 4.53 shows the effect of the 10_12

gradient, and gradient curve 7, along with 60/40 gradient elution on the chromatogram.

Table 4.13 Number of Resolved Peaks for RB 21 Optimization

Method	# of Peaks
10_12C7	38
10_12 and 60/40 elution	36
10_12C7 and 60/40 elution	36

Figure 4.51: C. I. Reactive Blue 21 Atlantis (C18) with 10_12 Gradient Time, 70/30 Elution, and Curve 7

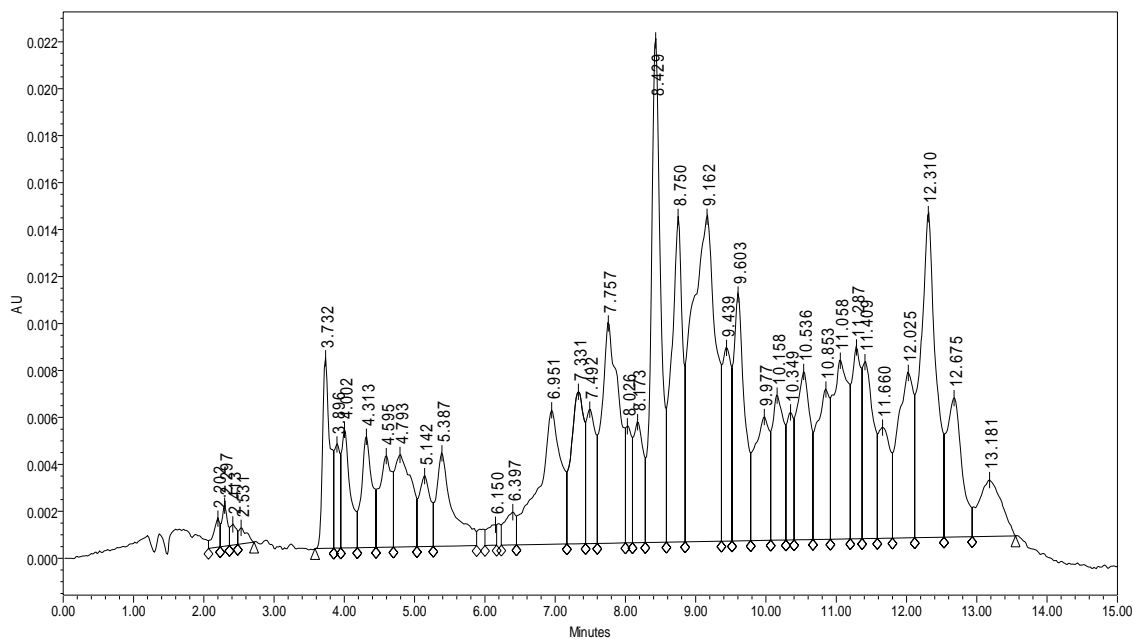


Figure 4.52: C. I. Reactive Blue 21 Atlantis (C18) with 10_12 Gradient Time, 60/40 Elution, and Curve 6

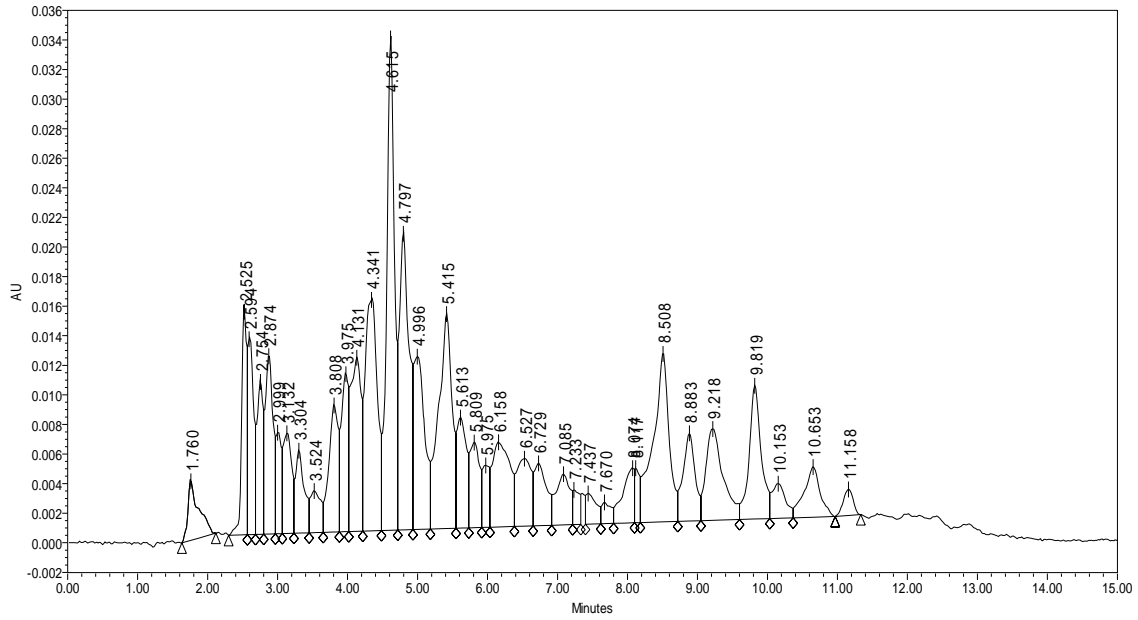
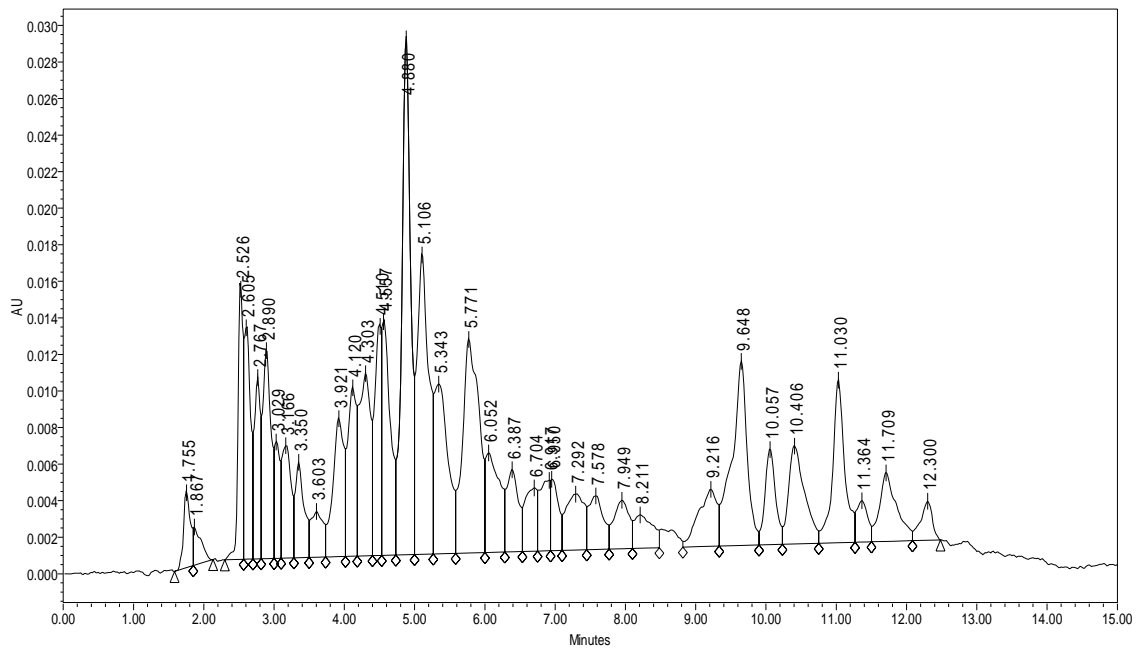


Figure 4.53: C. I. Reactive Blue 21 Atlantis (C18) with 10_12 Gradient Time, 60/40 Elution, and Curve 7



By combining the three gradient factors, surprisingly, improved chromatography is not achieved. The number of resolved peaks decreased slightly for all of the combined factor chromatograms. The addition of the curve to the optimized gradient time (Figure 4.51) improves separation of those peaks in the beginning of the chromatogram from those found in the end. This spreads out the late-eluting peaks. The gradient elution (Figure 4.52) seemed to give slightly improved the peak shape, and Figure 4.53 is a combination of both factors together. Though some slight benefits can be seen in peak shape for Figure 4.52, the decrease in the number of peaks overall make these combination factors not as optimal, as the gradient time changes solely. Thus, the optimal chromatogram for this dye considering time can be seen in Figure 4.46.

4.3 Reactive Blue 21 Hydrolysis and Dyeing Experiments

The complex structure of Reactive Blue 21 made analysis of this dye via HPLC extremely difficult. However, with the improved gradient system (Table 3.7) for Reactive Blue 21 on the Atlantis column, the number of peaks resolved was significantly increased. Without the use of advanced analytical techniques, such as HPLC-MS, identification of these peaks is difficult. However, by utilizing simple reactive dye experiments, general information about Reactive Blue 21 can be obtained. Hydrolysis, exhaust dyeing, and pad batch dyeing were used to determine if any of the peaks could be attributed to sulfatoethylsulfone (SES), vinyl sulfone (VS), or hydroxyethylsulfone (HES) forms of the commercial dye. All experiments were run using the optimal gradient.

4.3.1 Hydrolysis of Reactive Blue 21

Figures 4.54, 4.55, 4.56, and 4.57 are chromatograms obtained from aliquots of the Reactive Blue 21 hydrolysis bath. Figure 4.54 is the chromatogram of the solution prior to adding the dye to base, Figure 4.55 is the chromatogram of the solution after 10 minutes at 65°C, Figure 4.56 is the chromatogram at 25 minutes, and Figure 4.57 is the chromatogram at 50 minutes.

Figure 4.54: C. I. Reactive Blue 21 Prior to Adding Base for Hydrolysis

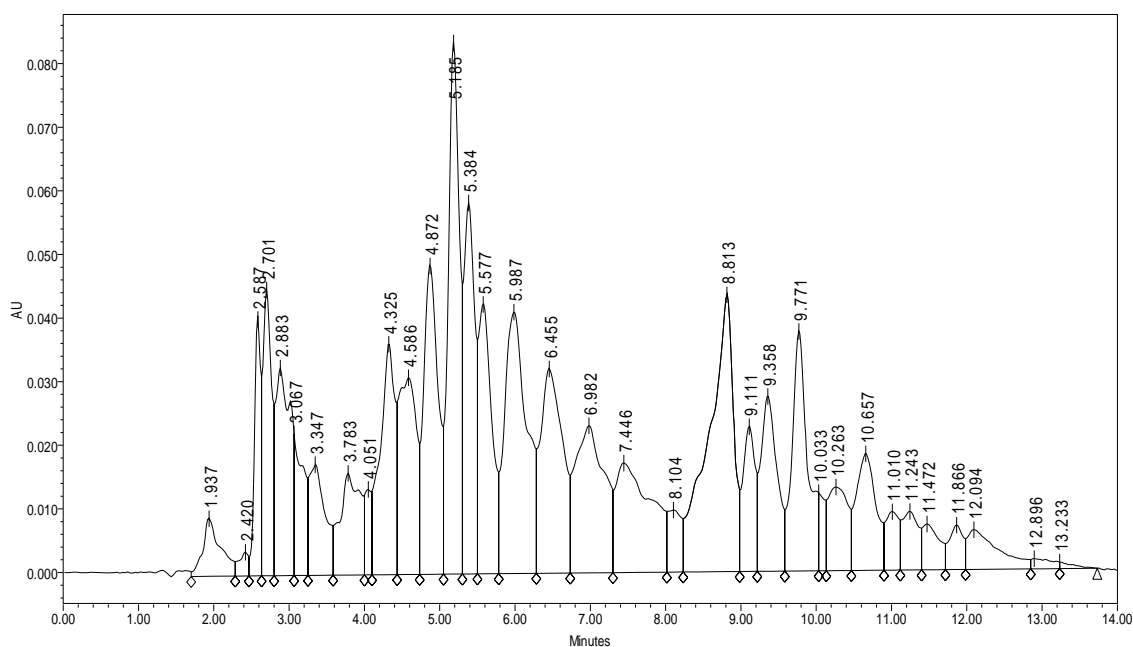


Figure 4.55: C. I. Reactive Blue 21 Hydrolysis at 65°-10 Minutes after Base was Added

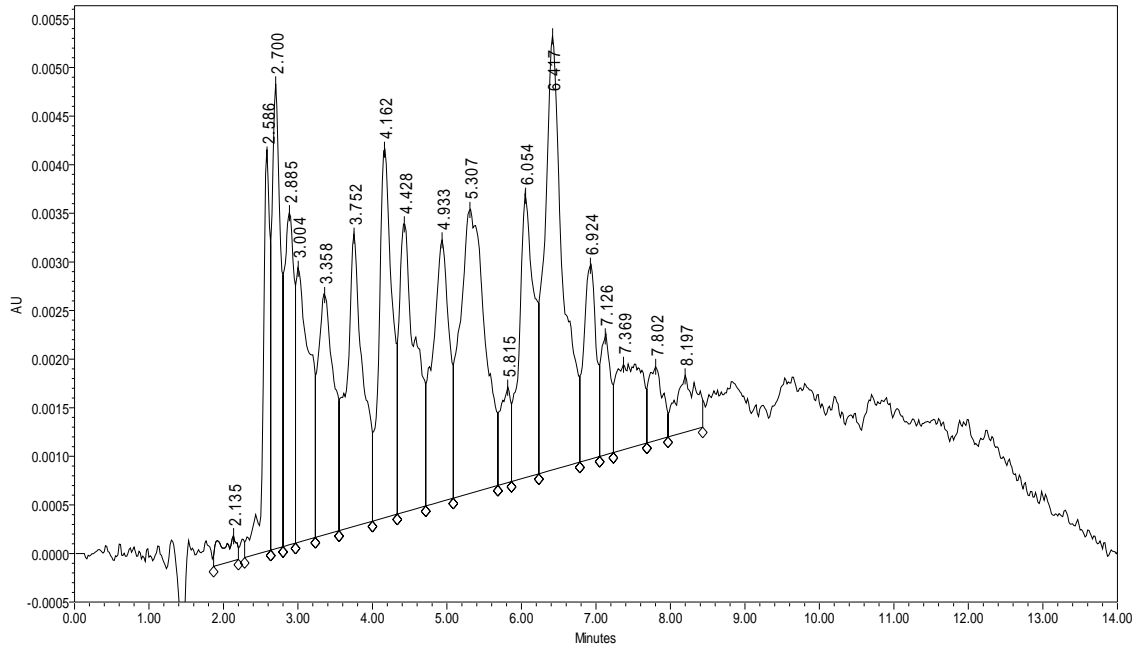


Figure 4.56: C. I. Reactive Blue 21 Hydrolysis at 65°-25 Minutes after Base was Added

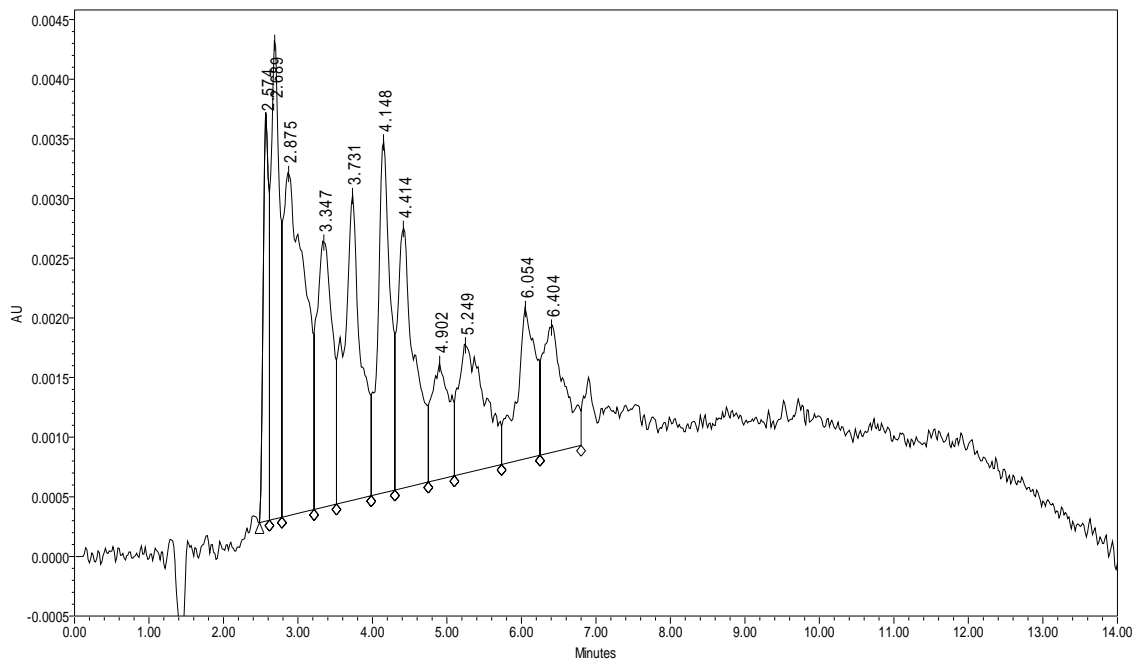
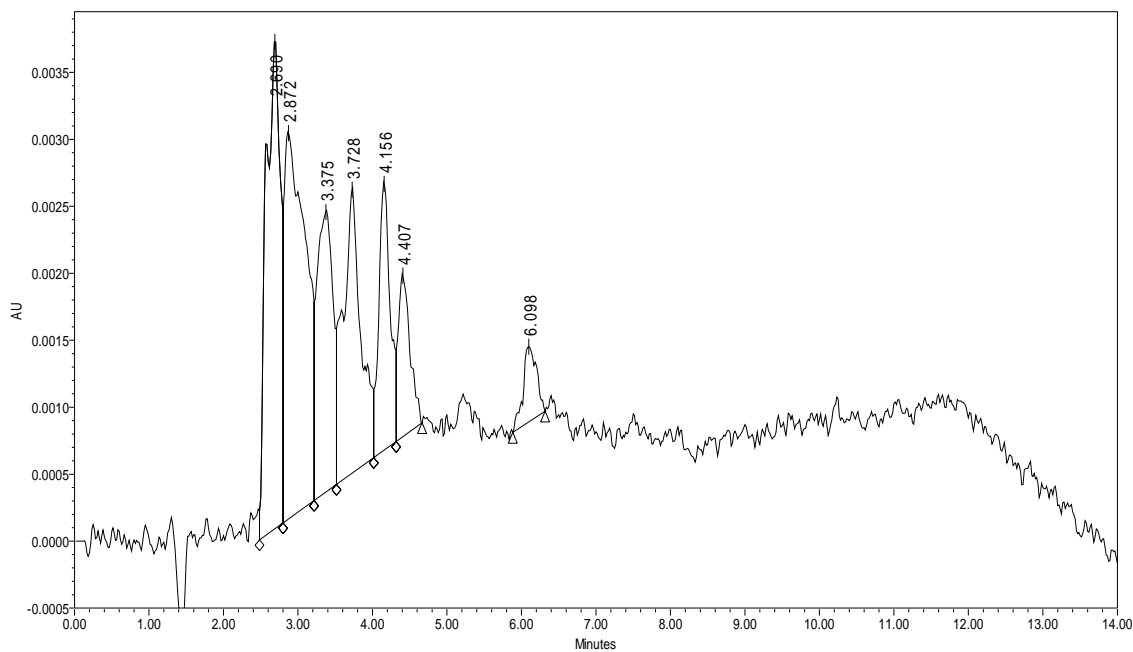


Figure 4.57: *C. I. Reactive Blue 21 Hydrolysis at 65°-50 Minutes after Base was Added*



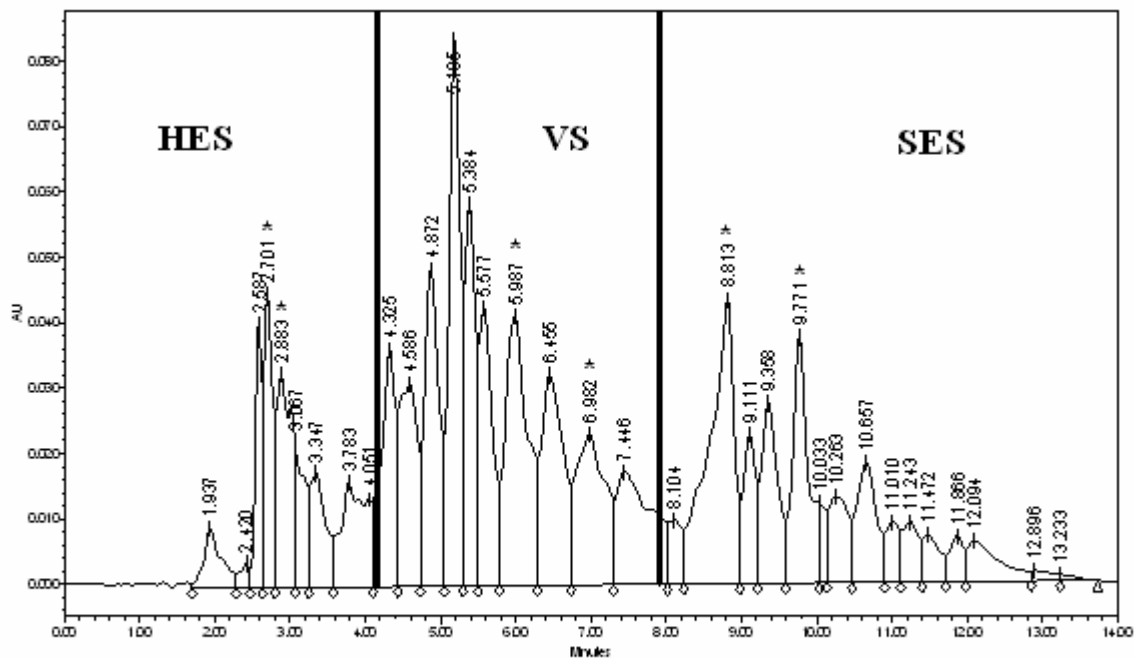
Figures 4.54, 4.55, 4.56, and 4.57 show that as hydrolysis continues, the peaks become more unsymmetrical and decrease from the left to the right side of the chromatograms. Table 4.14 shows the percent area of different peaks selected from Figure 4.54. Some of these peaks can be seen in all 4 chromatograms, while others are seen in a few, and some only in Figure 4.54. The peaks of interest were chosen from Figure 4.54 in different areas of the chromatogram based on the simplicity of tracking the particular peaks throughout the hydrolysis process.

Table 4.14 Peaks of Interest for Reactive Blue 21 Hydrolysis

Time of Hydrolysis	%Area			
	Before Alkali	10	25	50
Peaks of Interest				
2.7	3.02	6.07	9.6	22.66
2.9	3.75	4.91	17.32	26.52
4.4	N/A	7.38	10.33	6.28
6	7.06	6.54	6.54	3.29
6.9	5.17	3.68	N/A	N/A
8.8	8.31	N/A	N/A	N/A
9.8	4.54	N/A	N/A	N/A

Examination of the data in Table 4.14 shows that peaks (2.7 and 2.9) that are eluted from the column at the earliest time continue to increase in area as the time of hydrolysis increases. This is characteristic of HES, which is the hydrolyzed form of the dye. As hydrolysis progressed, the vinyl sulfone (VS) form reacts with water to produce the HES, and this accounted for the increase in percent area. Peaks that appear in the far right of the chromatogram (8.8 and 9.8) disappear fairly quickly during the experiment and are the SES form of the dye. This indicates that the dye has reacted with the alkali to produce the VS. This can explain why peaks like 4.4, did not show up in the initial chromatograms, but later appeared. That peak, as well as others (6 and 6.9), are probably the VS form of the dye. These peaks decrease in area as the HES form increases. Sometimes a slight increase in VS form for this dye can be seen, due to the effect of the initial addition of the base. Figure 4.58 shows a proposed grouping of peaks that represent HES, VS, and SES from Figure 4.54. Stars in the chromatogram above peaks denote peaks of interest.

Figure 4.58: Hydrolysis of C. I. Reactive Blue 21 Predicted Dye Forms



4.3.2 Exhaust Dyeing with Reactive Blue 21

Figures 4.59, 4.60, and 4.61 are the exhaust dyeing chromatograms of Reactive Blue 21.

Figure 4.59 is before Na_2CO_3 is added, Figure 4.60 is after the Na_2CO_3 is added and dye bath temperature reached 60°C , and Figure 4.61 is taken 40 minutes later than Figure 4.60.

Figure 4.59: C. I. Reactive Blue 21 Prior to Adding Base for Exhaust Dyeing

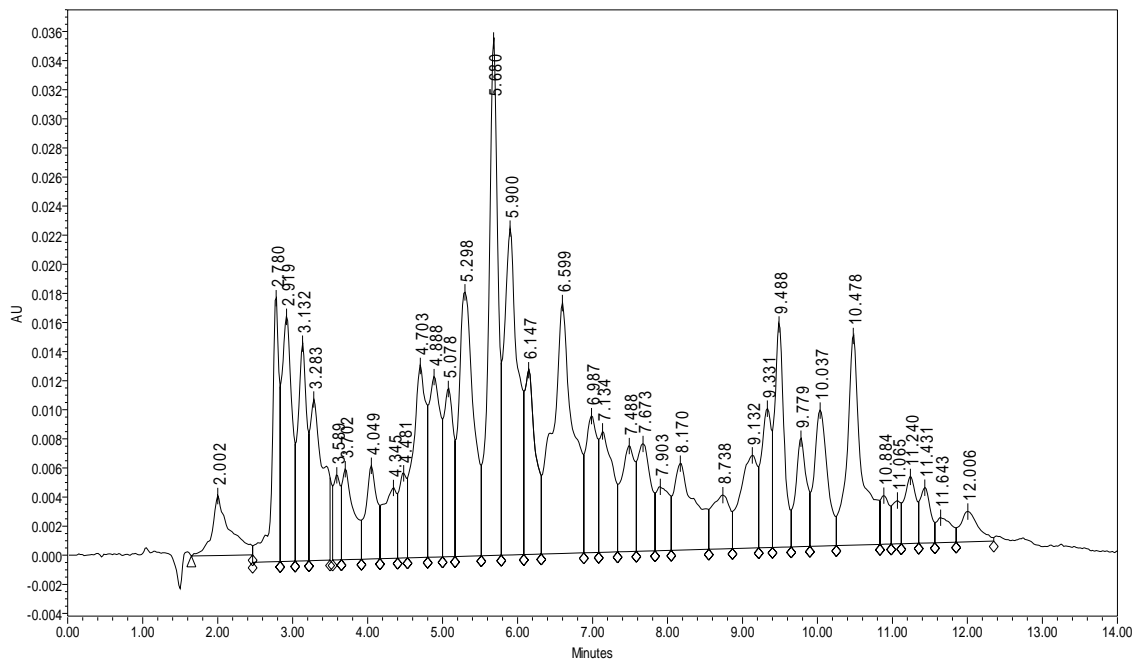


Figure 4.60: C. I. Reactive Blue 21 Exhaust Dyeing after Na_2CO_3 Addition

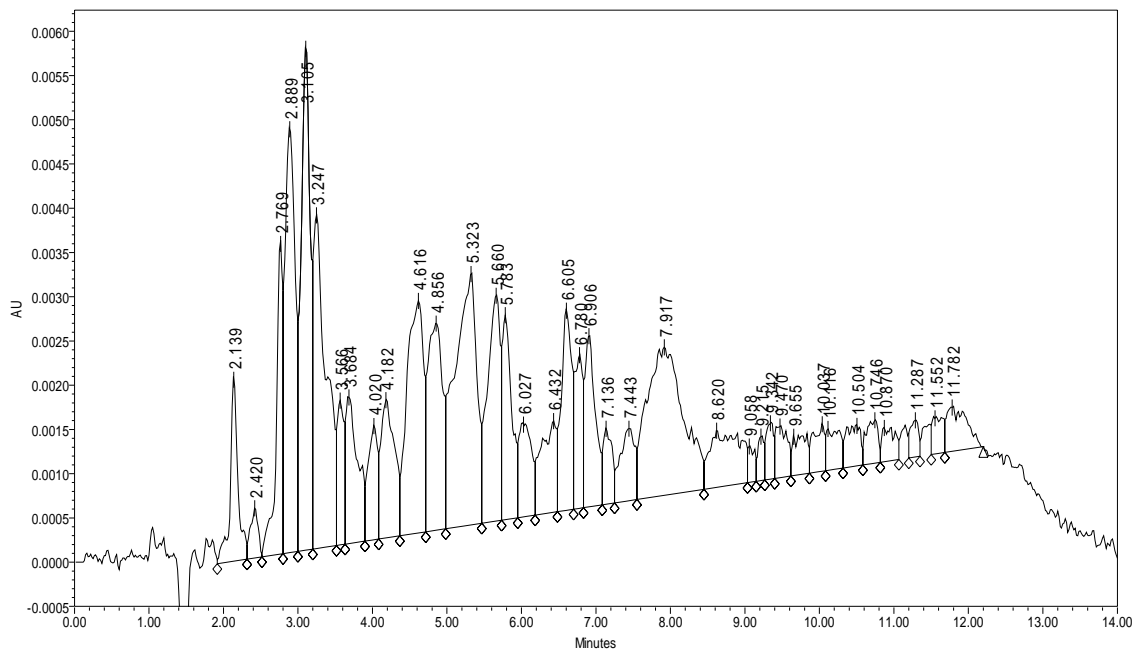


Figure 4.61: C. I. Reactive Blue 21 Exhaust Dyeing-40 Minutes after Na₂CO₃ Addition

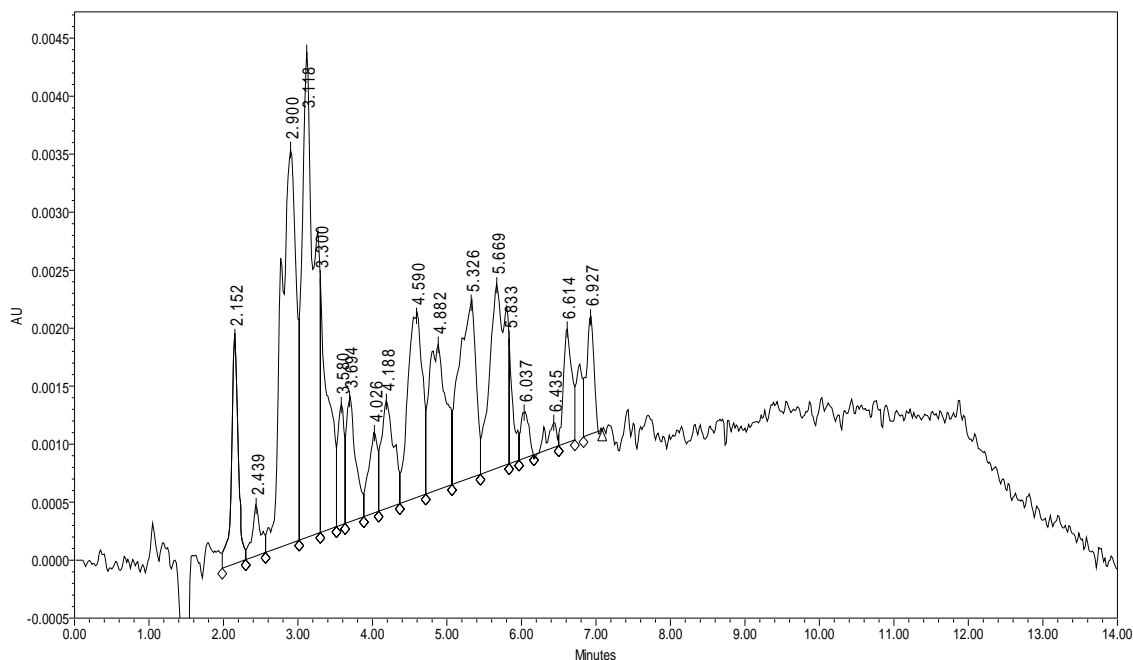


Table 4.15 (below) shows peaks of interest from the exhaust dyeing experiment. These peaks were chosen in the same manner as those in the hydrolysis experiment. These peaks are in the same relative position as those in the hydrolysis experiment. In Table 4.15, certain peaks (2.9, and 3.1) continue to increase during the dyeing, and represent the HES form of the dye, while other peaks represent the VS form (6.6 and 7.4), or the SES form (9.5 and 10.5). While peak 4.2 does increase slightly in the Table 4.13, it is believed to represent the VS form as well. During exhaust dyeing, hydrolysis is slightly slower, because the dyeing temperature is 5°C lower than in the hydrolysis experiment, but the results mimicked the general trend that is seen in peaks of the hydrolysis experiment. The absorbance values decreased by approximately ten-fold, indicating the expected loss of dye from the solution.

However, hydrolysis during the dyeing is an irreversible, unwanted reaction, and ideally should be limited [21, 26].

Table 4.15 Peaks of Interest for C. I. Reactive Blue 21 Exhaust Dyeing

	%Area		
Time of Hydrolysis	Before Alkali	0	40
Peaks of Interest			
2.9	3.85	6.91	17.3
3.1	3.02	3.17	17.75
4.2	N/A	3.01	3.93
6.6	8.08	3.28	2.78
7.4	2.23	3.91	N/A
9.5	3.24	0.97	N/A
10.5	4.84	0.95	N/A

N/A = no peak

4.3.3 Pad Batch Dyeing of Reactive Blue 21

Figures 4.62 and 4.63 are chromatograms from the Reactive Blue 21 pad-batch dyebath. Figure 4.62 is the chromatogram of the dye before adding the alkali solution while Figure 4.63 is from the dyebath the next day. In general, the chromatograms for the pad batch dyeing are similar to the chromatograms found at the beginning and end of hydrolysis and the exhaust dyeing.

Figure 4.62: C. I. Reactive Blue 21 Prior to Adding Base for Pad Batch Dyeing

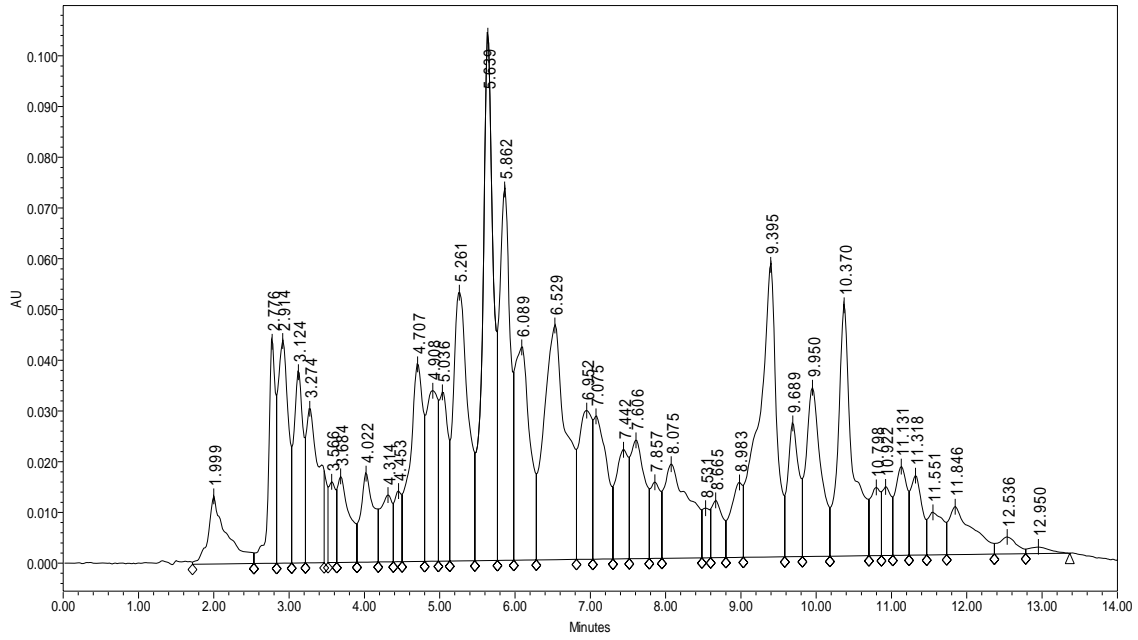
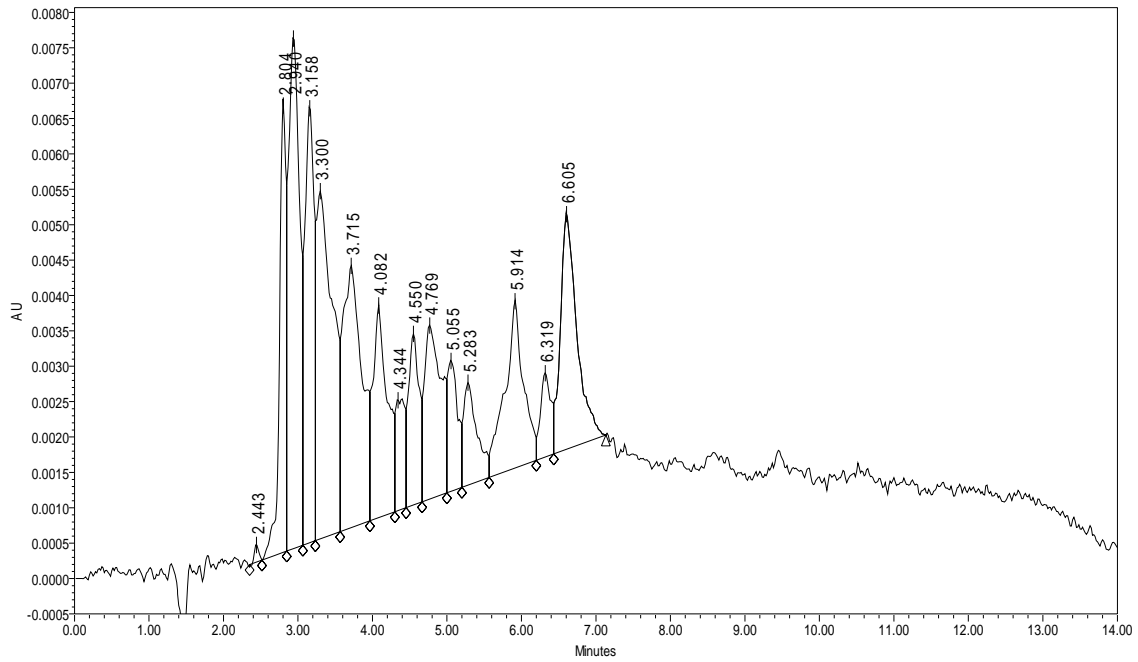


Figure 4.63: C. I. Reactive Blue 21 Pad Batch Dyebath (next day)



5. CONCLUSIONS

The two ion-pair reagents, TBAB and TBAHS, gave similar chromatograms for the reactive dyes in this study. However, these results were directly dependent on the column utilized. When the Atlantis column was employed, particularly for Reactive Blue 21, the TBAB gave superior results to the TBAHS. Therefore, it is recommended that for the analysis of reactive dyes, particularly Reactive Blue 21, when employing ion-pair chromatography, TBAB is the better IPA.

From stationary phase experiments on these dyes, it was found that adequate carbon loading was needed to effect separation. Phenyl (C_6H_5), and octadecyl (C_{18}) columns in the experiment provided adequate loading for all of the dyes, but the methyl (C_1) and butyl (C_4) columns did not. Neither column length nor particle size played the most important role in the separation of these dyes. Very similar results are found for columns of the same stationary phases with different lengths, and column packings, despite the fact that gradient used for analysis was based on the length of the column. The most important factor seemed to be column chemistry. The column that gave the best overall results for the dyes in this study was the Atlantis column, followed by the Exsil ODS column (both C_{18}).

Most dyes were analyzed well on the Atlantis column using Gradient 1, but Reactive Blue 21 is more complex than the others, and was further studied in order to improve analysis of it. The factors considered were gradient curve, gradient elution and time separately and then combined. Separately, improvements were shown when utilizing curve 7, when adjusting the gradient elution to 60/40, and by stretching the time taken for the initial

gradient to change for the analysis of Reactive Blue 21. Adjusting the gradient time yielded the best improvement in number of separated peaks. However, by decreasing the gradient slope, the run time is significantly increased. Taking this into consideration, the optimal method selected for gradient time was the 10_12 gradient. Despite improvement made by adjustments to the gradient curve and gradient elution, these effects are best shown separately. The improvements made separately were combined in order to determine if these factors could be optimized further. The combination of these factors actually reduced the number of peaks separated, so the optimal method for Reactive Blue 21 was still the 10_12 gradient.

In the Reactive Blue 21 hydrolysis and dyeing experiments (exhaust and pad-batch), the progression of hydrolyzed dye was examined to determine if the peaks of Reactive Blue 21 chromatograms could be attributed to the sulfatoethylsulfone (SES), vinyl sulfone (VS), or hydroxyethylsulfone (HES). The results for all three experiments were very similar, and those peaks that could possibly be attributed to the SES, VS, and HES forms of the dye were shown in Figure 4.58. Generally, those peaks that were retained later on the column (8.0 minutes and later) are possibly the SES form of the dye, those in the middle (between 4.1 and 8.0 minutes) could be the VS form, and those that are shown in the chromatogram first (earlier than 4.1 minutes) seem to be the HES form of the dye.

6. RECOMMENDATIONS FOR FUTURE WORK

One area that could be examined further is to develop more detailed analysis of Reactive Blue 21. By using hydrolysis and the two dyeings, hypotheses were made about which peaks represented the different forms of the dye. However other techniques such as LC-MS or even CE-MS, would provide detailed information as to what each peak really is. In order for this to be done however, more method development must take place considering that CE is a different technique, and the use of ion-pair chromatography for the LC must be either re-examined or abandoned, since studies have previously shown that IPAs such as TBAB or TBAHS produce very low analyte detection for certain detectors in MS [37].

Another area of consideration would be to examine other types of stationary phases, like C₈, as well as examining more application-specific designed columns to determine if the separation achieved in this study could be even further improved.

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