

**Monitoring for Sulfonated Azo Dyes
and Their Degradation Products
in Industrial and Municipal Wastewater**

by

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(ABSTRACT)

Sulfonated azo compounds are one of the most commonly used textile dyes. They pose an environmental problem because they can be difficult to degrade in municipal and industrial treatment systems and can cause effluent wastewaters to violate color standards. Methods were developed to monitor for three reactive azo dyes: Remazol Black B, Remazol Red RB, and Remazol Golden Yellow 3RA, and their degradation products. HPLC/diode array detector, GC/MS, and LC/MS were utilized to characterize the purified dyes and the wastewaters and identify degradation products of the dyes resulting from several biological and chemical treatment processes. Results indicate that chemical reduction and anaerobic biological treatment of the dyes both result in cleavage of the azo bonds and formation of aromatic amines such as p-(2-hydroxyethylsulfone)-aniline, a reduction product of all three dyes. Some of these amines were unstable and quickly reacted to form other chemical structures. The textile mill effluent contained about thirty organic compounds detectable by GC/MS,

including p-(2-hydroxyethylsulfone)-aniline. Anaerobic treatment caused little degradation of these compounds, but aerobic treatment degraded most of them. This research only investigated wastewater from a textile facility which performed pad dyeing of the cotton in a cotton/polyester blend. The sulfonated azo dyes used were reactive dyes typically applied to cotton fabrics.

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CHAPTER I

INTRODUCTION

Dyes comprise one of the largest classes of industrial chemicals manufactured, with over 245 million pounds produced in the United States in 1980 and another 29 million imported (Baughman and Perenich, 1988). Most of the dyes are applied to textiles, food, or plastics, but inevitably some dyes are released from industrial processes and into receiving waters, sediments, and soils. The sheer volume of chemical involved would justify the investigation and control of dyes released to the environment as a toxicological concern. However, the ability of dyes to impart color makes even relatively low concentrations of dyes highly visible in the environment, and thus, also poses an aesthetic concern to those who use dye-contaminated water as a source of drinking water or recreation.

Azo compounds used in the textile industry comprise the largest class of dyes, accounting for 60 - 70% of the total dyes produced (Richardson, 1988). The stability of these compounds, evidenced by high light fastness and resistance to reaction with acids, bases, and detergents, also makes them difficult to degrade in conventional wastewater treatment facilities, and can cause receiving waters to violate color

standards. With these problems in mind, research was undertaken to investigate methods of treating a textile dye wastewater containing three sulfonated, reactive azo dyes.

Traditional methods of evaluating the effectiveness of a wastewater treatment process involve the measurement of such parameters as BOD, TOC, or COD. However, to understand the fate of individual compounds, such as dyes, it is necessary to develop analytical methods for detecting and quantifying them in environmental samples. In addition, it is valuable to identify the degradation products resulting from various treatment methods. This is especially important with azo dyes, which can degrade to form compounds (e.g. aromatic amines) which may be more toxic than the dyes themselves. Sulfonated azo dyes present an analytical challenge due to their large size and polarity, which require the application of specialized chromatographic and mass spectrometric techniques.

Thus, the objectives of this research were threefold:

- (i) To develop methods for monitoring and quantifying three sulfonated azo dyes in aqueous samples
- (ii) To detect and identify degradation products of the dyes
- (iii) To apply these techniques to treated and untreated wastewater samples.

CHAPTER II

LITERATURE REVIEW

Synthetic Organic Dyes

The organic chemical industry was born in 1856 when Sir William Henry Perkin, a student at the Royal College of Chemistry in London, founded a company at the age of 18 to manufacture a mauve dye of his own discovery (McMurry, 1988). Since that time, the dyestuffs industry has expanded and thrived, with dyestuffs accounting for a large percentage of all synthetic organic chemicals produced in the United States. The need to dye all types of substances, such as cotton, nylon, polyester, food, plastics, etc., in virtually any color has resulted in a huge variety of dyes, many with little in common chemically except the ability to impart color. However, most all organic dyes impart color due to shifts in electron energy.

The Origin of Color in Organic Molecules

When electromagnetic radiation strikes an organic molecule, some of the energy of the light is absorbed by the

molecule and used to excite electrons from lower energy bonding molecular orbitals to a higher energy anti-bonding orbitals. However, not all wavelengths of radiation are absorbed equally by the compound. Only those wavelengths which provide the right amount of energy to make the transition from the bonding to anti-bonding orbitals are absorbed. The resulting absorption spectrum of an organic compound (Figure 1) shows that an organic compound absorbs radiation in a characteristic way, absorbing certain wavelengths or ranges of wavelengths, and reflecting those wavelengths which it does not use. Planck's law states that the wavelength of electromagnetic radiation is inversely proportional to it's energy. The law has the mathematical form

$$E = hc / \lambda$$

where E is energy, c is the speed of light, lambda is wavelength, and h is Planck's constant which has a value of 6.62618×10^{-34} J s. This important relationship indicates that the less energy it takes to excite an electron of an organic compound, the higher wavelength of radiation absorbed.

Most simple organic compounds, such as aliphatic alkanes, alcohols, and carboxylic acids, absorb radiation in

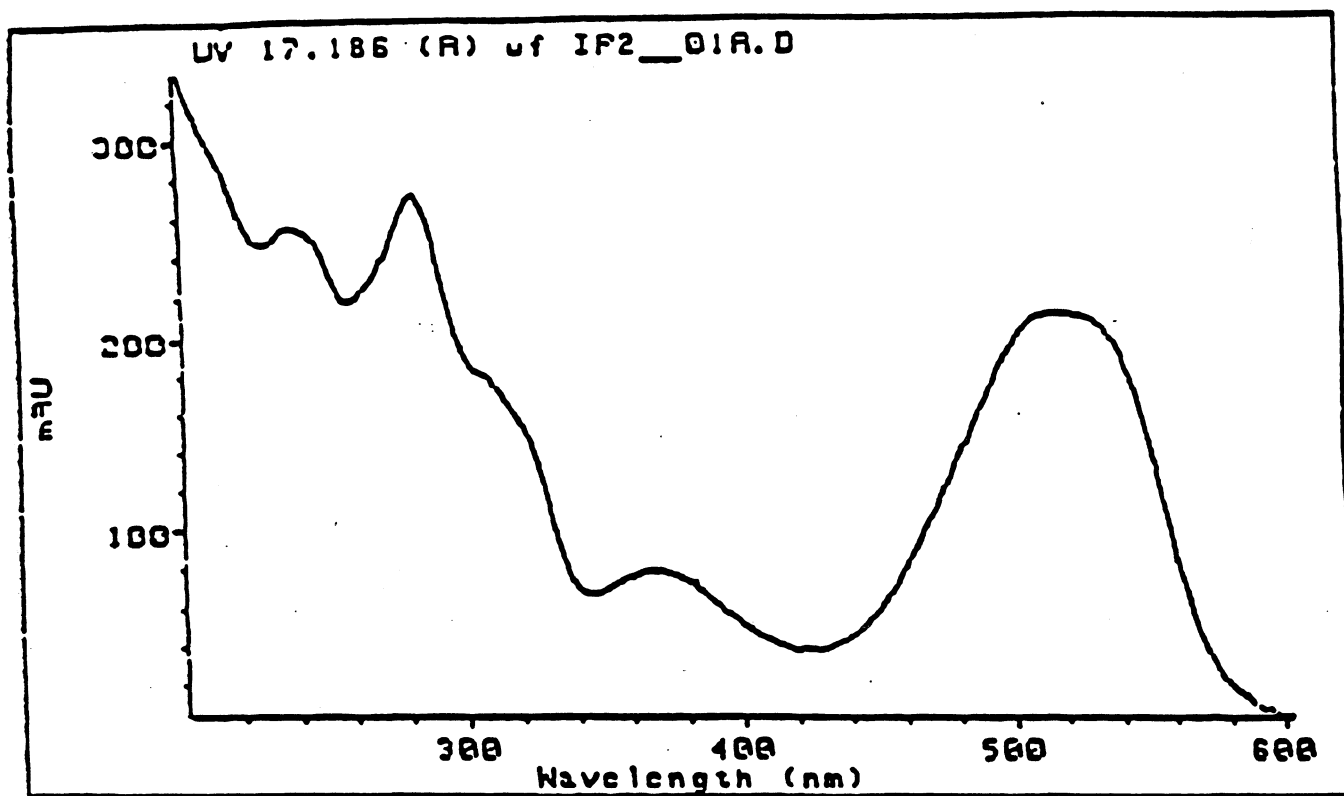


Figure 1: UV/Visible absorbance spectrum of Remazol Red RB, a reactive azo textile dye.

wavelengths below the visible range of 380 nm to 780 nm, and thus have no apparent color. Compounds with electrons in conjugated pi orbital systems, however, require less energy for excitation and thus absorb at a higher wavelength. A conjugated pi orbital system is formed when a molecule has alternating single and double bonds; i.e., a series of overlapping p orbitals which delocalize the electrons (McMurry, 1988). The larger the extent of the conjugation, the lower the energy transition from bonding to anti-bonding molecular orbitals and thus the higher wavelength absorbed.

Classification of Dyes

Dyes are commonly classified by structure, method of application, or fiber type (Abraham, 1968, Waring, 1990). None of these classification systems is satisfactory by itself, and two are often used simultaneously to describe a dye (e.g., reactive azo dye). Classified by structure, important dyes used in the textile industry include azo, anthraquinone, nitro and nitroso, sulfur, styryl, polymethine, benzodifuranone, phthalocyanines, quinophthalone, and indigoid dyes (Waring, 1990). Classified by method of application, important dye types include reactive, direct,

disperse, acid, basic, ingrain, mordant, and vat (Waring, 1990). It is beyond the scope of this work to define these numerous dye types. However, the structural class azo and the application class *fiber-reactive* will be described in more detail.

Azo Dyes

Azo dyes are one of the most commonly used classes of dyes, accounting for over 50% of all commercial dyes (Waring, 1990). The term "azo" refers to compounds which contain one or more nitrogen-nitrogen double bonds, usually connecting two aromatic systems. The vast majority of azo dyes are monoazo (containing only one azo linkage), but disazo dyes (containing two azo linkages) are also common (Waring, 1990). Dyes containing three, four, or more azo linkages also exist. The azo bond is *trans* in nature, and the bond angle is approximately 120 degrees (Waring, 1990).

Azo bonds are useful in creating colored compounds because the sp^2 -hybridized nitrogen atoms are able to extend conjugation between aromatic systems, which are themselves fully conjugated. By linking benzene-based, naphthalene-based, or even larger aromatic systems together, electrons are delocalized over a large molecule. Different combinations of aromatic systems, linked by azo bonds, can be created to

absorb energy at different wavelengths in the visible spectrum and thus reflect many different colors. In general, the larger the molecule, i.e., the more azo bonds, the more bathochromic the dye (i.e., longer wavelengths are absorbed).

Azo dyes are synthesized by a two step process. In the first step, a primary aromatic amine is diazotized by reaction with hydrochloric acid and sodium nitrite (Figure 2). The resulting diazonium salt is a weak electrophile and thus will react with and be coupled to strong nucleophiles such as amines and hydroxy compounds. Because there is a large variety of primary amines and coupling components available to dye chemists, there is a huge variety of different azo dyes that can be invented to create most colors.

Azo compounds exhibit several forms of tautomerism, or existence as an equilibrium between two structures. This is important to dye chemists because the tautomers have different colors and fastness properties. Hydroxyazo dyes, synthesized from a primary aromatic amine and an aromatic alcohol, exhibit azo/hydrazone tautomerism in which the dye exists as a mixture of azo and hydrazone forms (Figure 3a). Azonaphthols and phenylazo naphthols, in particular, exist as the hydrazone form in high proportion (Zollinger, 1990). In disazo dyes prepared with aminonaphthols such as H-acid, one of the azo

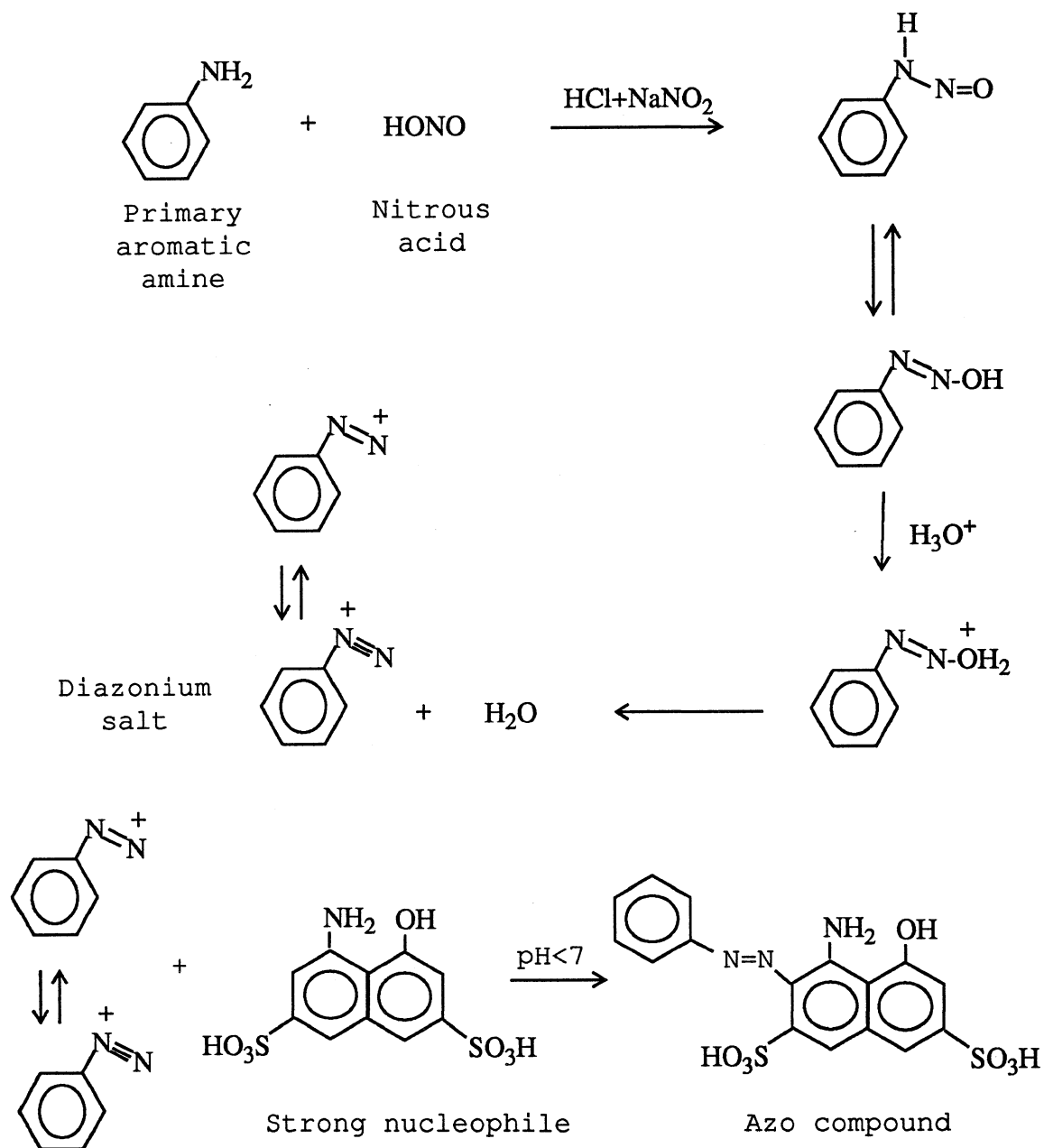


Figure 2: The two-step synthesis of azo compounds. A primary aromatic amine is first diazotized with HCl and NaNO2 and then coupled to a strong nucleophile.

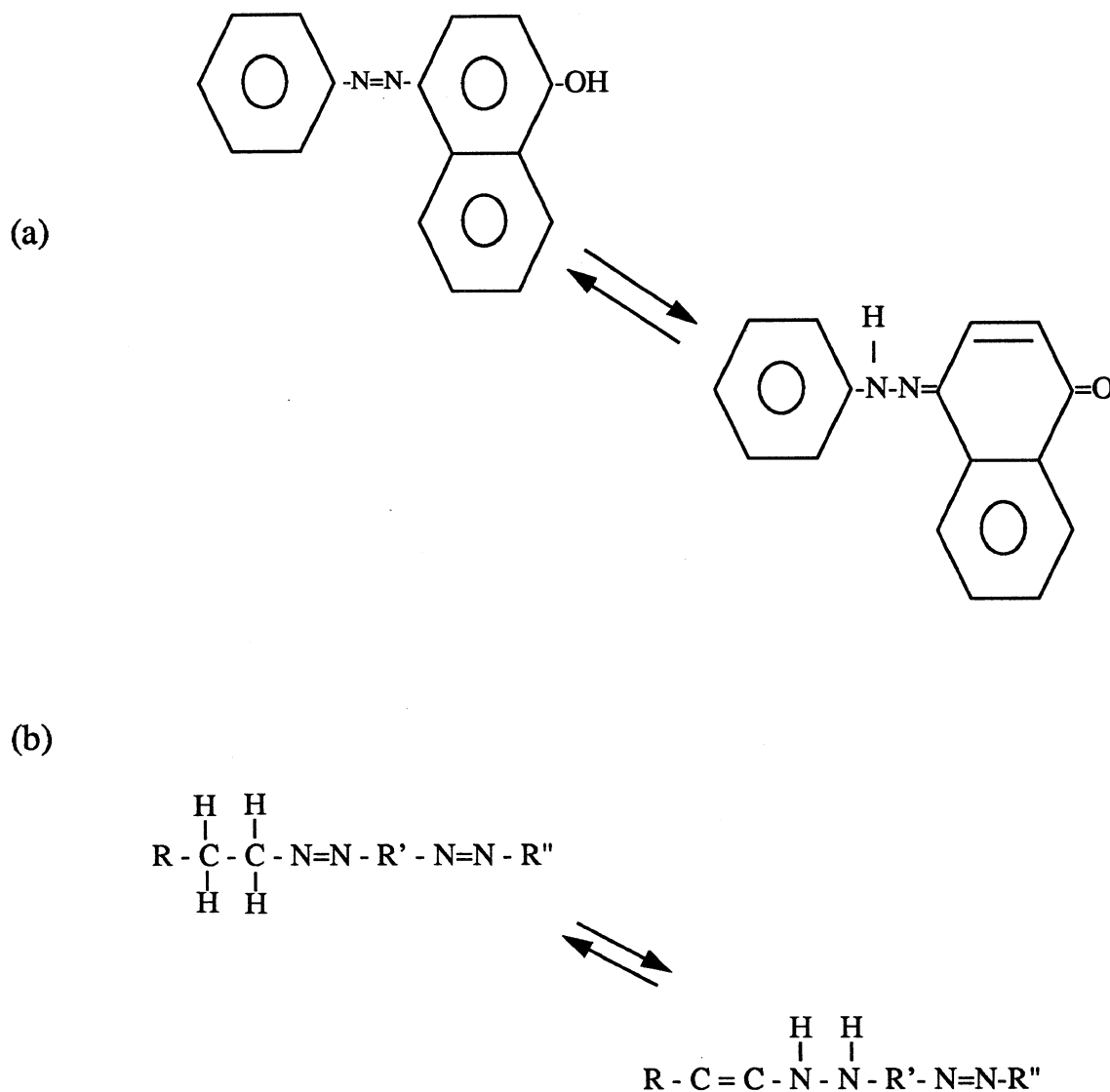


Figure 3: Tautomerism of azo compounds. (a) Azo/hydrazone tautomerism. (b) Azo/hydrazo tautomerism, in which one nitrogen-nitrogen linkage exists as a hydrazo bond.

bonds exists as a true azo bond while the other exists as a hydrazo bond (Figure 3b) (Waring, 1990).

The acid-base chemistry of azo compounds has made them useful as indicators, such as Congo Red, Methyl Orange, and Methyl Red. Protonation may occur at the terminal nitrogen of an aminoazo dye, or at the beta-nitrogen of the azo bond to give a resonance-stabilized azonium compound (Figure 4) (Waring, 1990). The azonium compound is usually brighter and absorbs at higher wavelengths than the non-protonated dye (Waring, 1990). In general, it is not desirable for azo dyes used in dyeing textiles to exhibit acid-base properties between pH 4 and 11 (Zollinger, 1991).

Reactions of Azo Compounds: The dye industry has extensively investigated azo synthesis reactions and the properties of azo dyes, but has paid little attention to further reactions of azo compounds (Zollinger, 1961). This is because azo compounds are very stable end products of manufacturing, and are rarely used to synthesize other chemicals. For these reasons relatively little is known about the reactions of azo dyestuffs.

One reaction that has been investigated and that also has implications for the fate of azo compounds in the environment is reductive cleavage of the azo bond (Figure 5). Strong

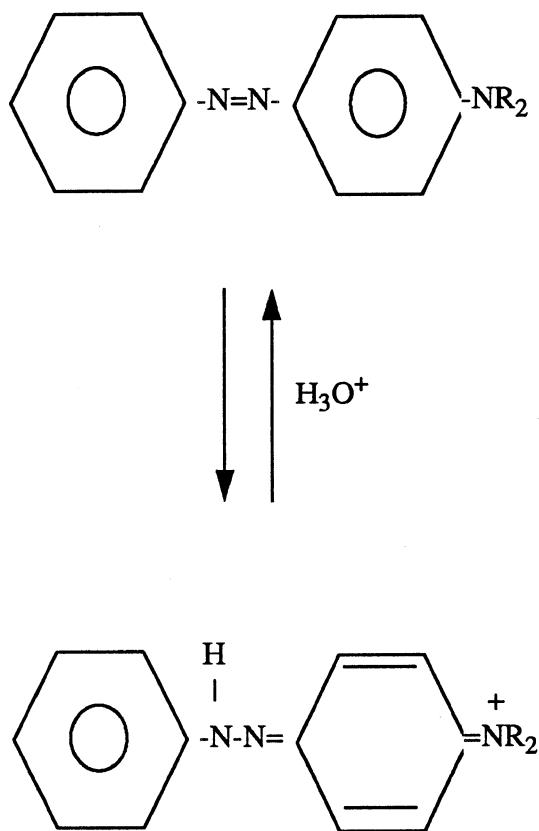


Figure 4: Formation of a resonance-stabilized azonium compound by protonation of the β -nitrogen of the azo bond.

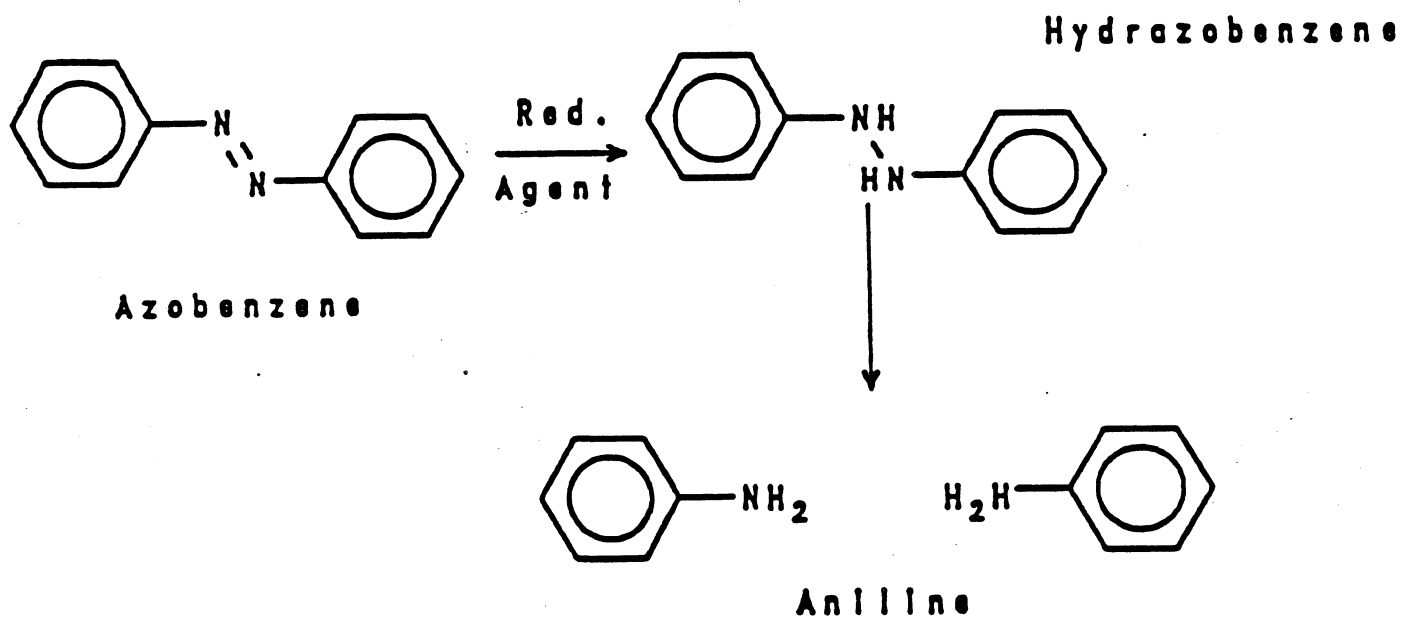


Figure 5: Reductive cleavage of the azo bond.

reducing agents such as sodium hydrosulfite or sodium borohydride cause addition at the azo bridge and the formation of two aromatic amines (Zollinger, 1961). The hydrazo compound is an intermediate to this reaction, but it is very difficult to isolate unless a milder reducing agent is used such as hydrogen sulfide (Zollinger, 1961). Much evidence suggests that certain microbes can perform this reduction biochemically in an anaerobic environment (Brown and Hamburger, 1987; Gardner et al., 1990).

The reductive cleavage of the azo bond can be exploited for dyeing, analytical, or treatment purposes. For example, on some textiles a pattern may be printed with a paste containing a strong reducing agent, and when the fabric is dyed and steamed, the azo dyes will be reduced on the printed part and the pattern will show up white on a colored background (Zollinger, 1961). As will be seen, chemical and biological reduction of azo dyes provide effective methods of removing azo dyes from industrial textile wastewaters.

Fiber Reactive Dyes

Fixation of textile dyes onto fibers may be accomplished in several different manners, including attachment by van der Waals forces, hydrogen bonding, salt links, or physical enmeshment (Laptev, 1973, Beech, 1970). However, the

strongest attachment is achieved by using a reactive dye, which, as the name implies, reacts with cotton or wool fibers to form a covalent bond. Reactive dyes demonstrate superior fastness to vat, direct, acid, and other dyes (ranging from 50% - 90%), require less complicated dyeing equipment, and are more compatible with continuous (as opposed to batch) dyeing processes (Beech, 1970). Most reactive dyes are either azo or anthraquinone. Reactive dyeing on an industrial scale began in 1956 with the introduction of the Procion dyes of ICI, one century after Perkin's discovery of Mauveine (Beech, 1970). Two kinds of reactive dyes relevant to this research are those containing triazinyl groups, and those containing the vinyl sulfone group.

The Procion dyes used the triazinyl group, a reactive group which is still used in over 50% of the reactive dyes sold today (Waring, 1990). Triazinyl reactive dyes are prepared using cyanuric chloride (Figure 6), a highly reactive substance which is susceptible to nucleophilic attack by compounds containing amino, hydroxyl, or alkoxyl groups (Beech, 1970). The three possible displacements of chlorine on one cyanuric chloride molecule each occur at a specific temperature: the first displacement at 0-5 degrees C, the second at 35-40 degrees C, and the third at 80-85 degrees C

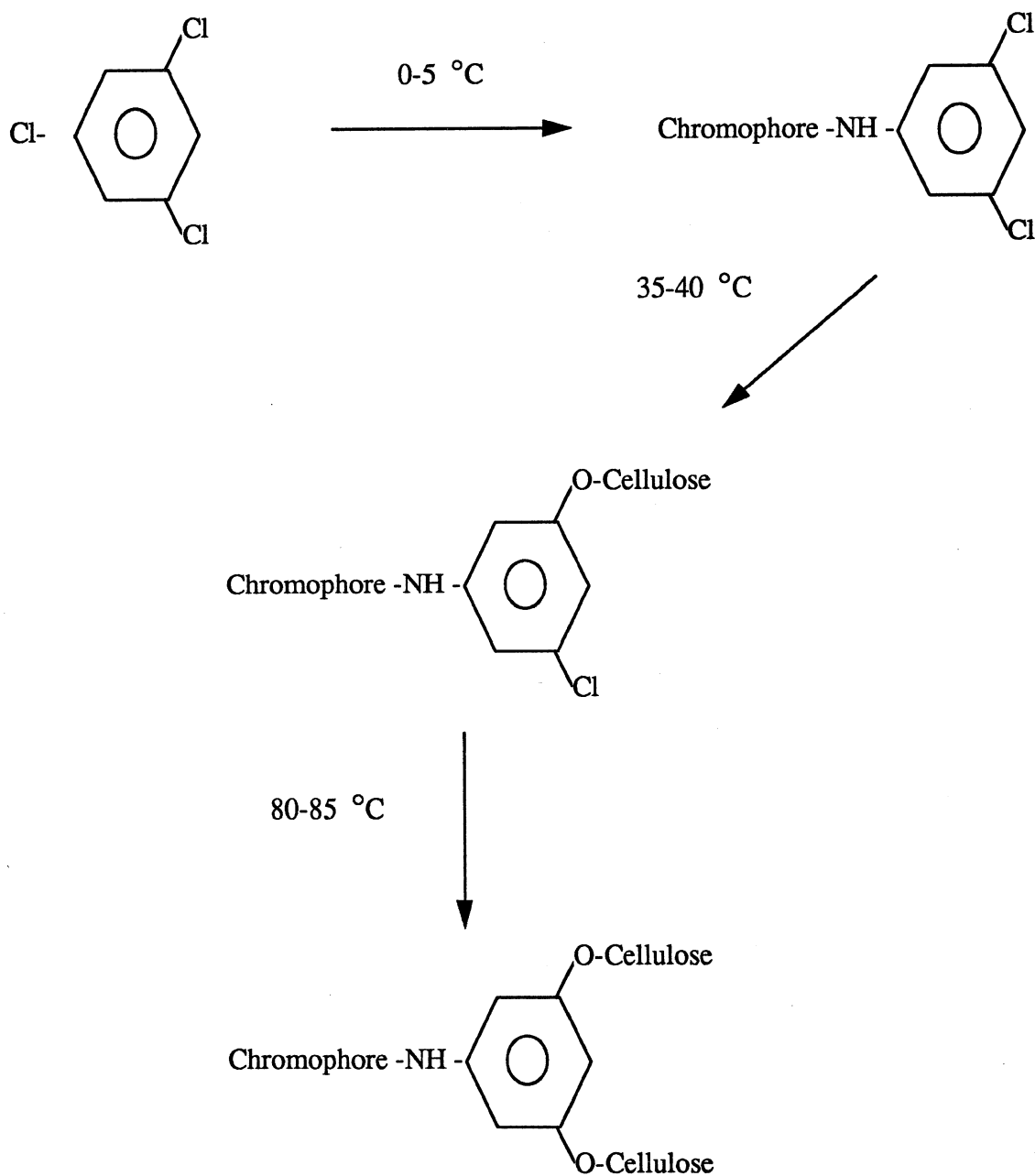


Figure 6: Nucleophilic substitution of chlorine atoms on cyanuric chloride.

(Waring, 1990). By mixing cyanuric chloride with an amino-containing chromophore in aqueous solution at 0-5 degrees C, a dye is formed that can react with hydroxy and amino groups on cellulose at 35-40 degrees C. Alternately, the second chlorine may be displaced by some other amine or hydroxide, forming a dye which will react with cotton at 80-85 degrees C (Abraham, 1968). Triazinyl reactive dyes are subject to hydrolysis, as water can nucleophilically replace a chlorine.

In 1958, only two years after the introduction of the Procion dyes by ICI, Hoechst introduced its own line of fiber-reactive dyes known as Remazol dyes. The Remazol reactive group is stored and transported as the sulfate ester of hydroxyethylsulfone, but reacts under basic conditions to form a vinyl sulfone group (Figure 7). The vinyl sulfone group is susceptible to nucleophilic addition and may react with ionized hydroxy groups on cellulose to form a covalent ether linkage to cotton. Alternately, the vinyl sulfone group may undergo a hydrolysis reaction and be converted to a hydroxyethylsulfone group. Hydrolyzed dye may no longer react with cotton, but may act as an acid dye.

Sulfonated Dyes: Sulfonic acid groups are added to some dyes to increase their solubility in water and increase their acidity (Laptev, 1973). All reactive and acid

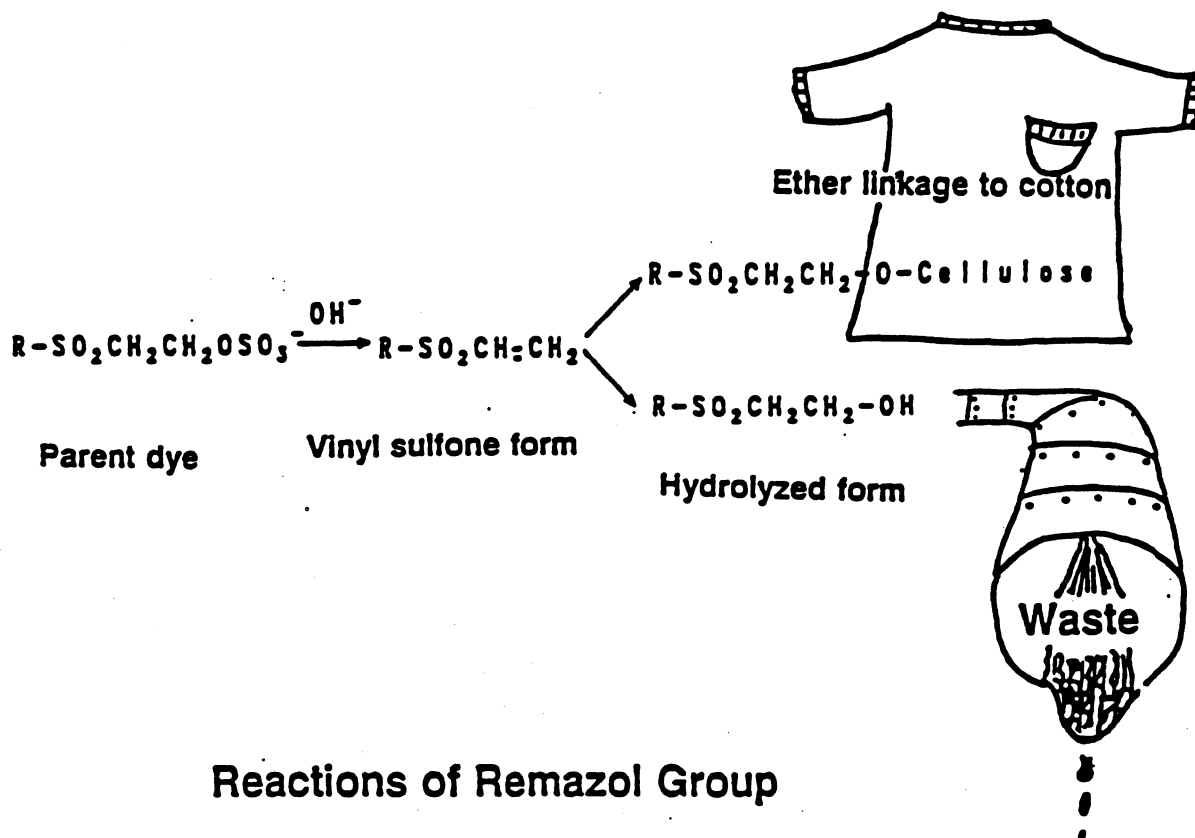


Figure 7: Reactions of the Remazol reactive group. The sulfatoethylsulfone form reacts at high pH to form a vinyl sulfone group, which can form an ether linkage with cotton or undergo hydrolysis.

dyes are sulfonated. Their extreme solubility make necessary the addition of high concentration of inorganic salts to the dye baths in order to "salt out" the dyes onto the fiber. The polar nature of sulfonated organic compounds makes them a special challenge with regard to chromatographic and mass spectrometric analysis: they are too polar/thermally labile for gas chromatography or GC/MS, and may require the use of ion-pair reagents with reverse phase LC analysis.

The Pad Dyeing Process and Navy 106 Dye Washwater

There are several commonly used industrial processes for the application of reactive dyes onto cellulosic fibers. Of particular interest to this research is the pad dyeing process, a semi-continuous process in which strips of fabric are conveyed through a hot dye bath. Excess dye is removed using pinch rolls, the fabric is held 12 to 24 hours in hampers until the reaction is completed, and then rinsed. The resulting wastewater which contains spent dyeing solution is known as the washwater, and is generally discharged to a local wastewater treatment facility.

One such wastewater is the Navy 106 Dye Washwater, produced from a textile plant in Martinsville, Virginia. The

Navy 106 dyeing solution is formulated to pad dye cotton and cotton/polyester blends, though the polyester fibers in the fabric are dyed by another process. It contains three major sulfonated azo dyes: Remazol Black B (70%), also known as Reactive Black 5, Remazol Red RB (26%), and Remazol Gold Yellow 3RA (4%), also known as Reactive Orange 16. The structures of Remazol Black B (Figure 8) and Remazol Gold Yellow 3RA (Figure 9) are non-proprietary, but the structure of Remazol Red RB is proprietary so that a hypothesized structure (Figure 10) is presented here. All three dyes are manufactured by the Hoechst-Celanese Corporation (Somerville, NJ), and include the vinyl sulfone reactive group. Remazol Red RB also includes a triazinyl reactive group.

In addition to the three dyes, other chemicals are added to the Navy 106 dye bath to improve fixation of the dyes onto the fibers. Sodium hydroxide is added to achieve the high pH (10.5-12) necessary to ionize hydroxy groups on the cellulose. Sodium chloride and sodium silicates are added to effect a "salting out" of the dye onto the fiber. Other additives may include surfactants such as pine oil, tributyl phosphoric acid, and isopropyl alcohol to reduce foaming, mild oxidants to prevent reduction of the dyes, and detergents such to aid in the rinsing of unfixed dye from the fiber. The actual

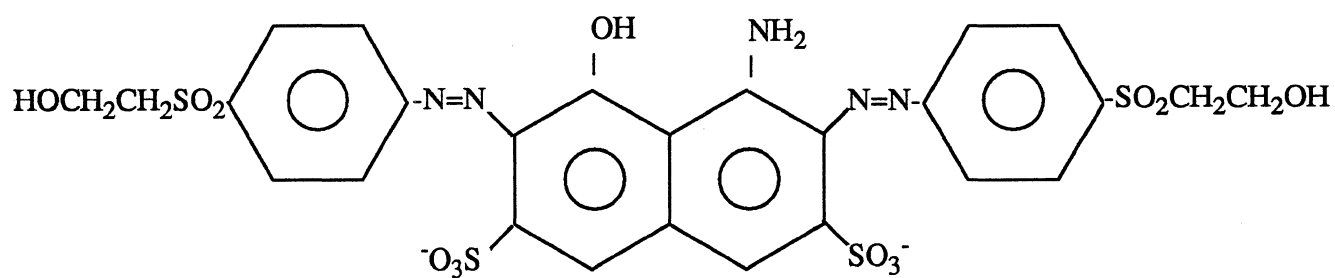


Figure 8: Structure of hydrolyzed Remazol Black B (Reactive Black 5).

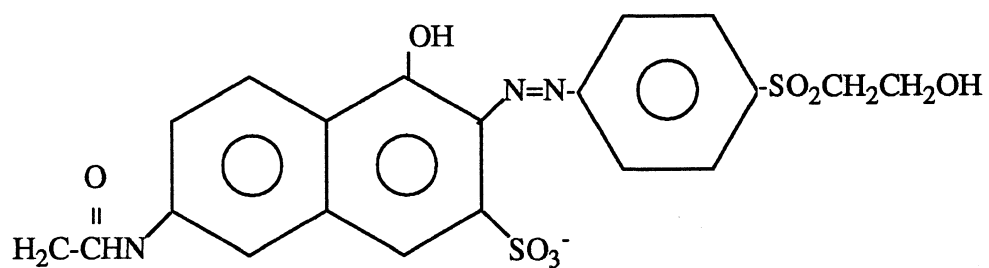


Figure 9: Structure of hydrolyzed Remazol Gold Yellow 3RA (Reactive Orange 16).

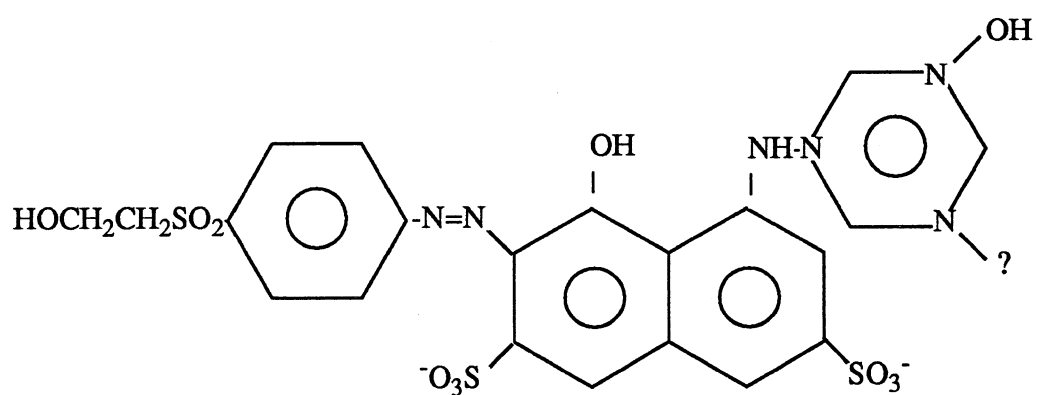


Figure 10: Hypothesized partial structure of hydrolyzed Remazol Red RB (Riefe, 1990).

concentrations of these additives are proprietary. The TOC of untreated Navy 106 Dye Washwater is about 350 mg/L (Loyd, 1992), only a few percent of which is due to dye (Powell, 1992).

Environmental Occurrence and Biodegradation of Dyes

In terms of mass produced per year, dyes comprise one of the largest classes of industrial chemicals. It is estimated that 245 million pounds of dyes were produced in the United States in 1980; another 29 million pounds were imported (Baughman and Perenich, 1988). Of this, approximately 12% was lost to wastewater streams during industrial dyeing processes, and of this, about 20% was discharged to receiving waters from wastewater treatment plants (Weber et al., 1990). Good dyestuffs are resistant to degradation from light, detergents, mild acids and bases, atmospheric pollutants, and dry cleaning solvents, and this resistance translates into a remarkable stability under many conditions encountered in the environment (ETAD, 1986; Loyd, 1992).

The most obvious negative effect that dyes have on a receiving waters is color. While this is mostly an aesthetic concern, it's high visibility makes it hard to ignore,

especially for those who must use the water as a source of drinking water. Dye toxicity to humans or to aquatic life, while a more serious concern, is generally low (Anliker, 1979). With the exception of some basic dyestuffs, few dyes have exhibited acute toxicity to fish and *Daphnia* at concentrations that would not make the dye highly visible in water (ETAD, 1986). Little is known about the chronic toxicity of dyestuffs. Similarly, few dyes have been found to bioaccumulate, evidenced by n-octanol/water partition coefficients less than 1000 in over 75 dyes investigated by member companies of ETAD (ETAD, 1986). Even disperse dyes with K_{ow} 's over 1000 tend not to bioaccumulate, probably because their tendency to aggregate inhibits transport across cell membranes (ETAD, 1986).

Many studies of the biodegradability of dyes have indicated that, with some exceptions, dyes are not degraded in aerobic biological treatment systems (Brown and Hamburger, 1987, Shaul et al., 1988, Gardner et al., 1990, Holdsworth et al., 1990, McCurdy, 1990, Loyd, 1992). However, many dyes are removed from the wastewater by sorption onto sludges (Games and Hites, 1977, ETAD, 1986, Shaul et al., 1990) Ganesh, 1992). The larger the aromatic system and fewer the sulfonic acid groups (i.e. the less water soluble) the greater sorption

of a dye onto biomass (Shaul et al., 1988). Dyes may also be sorbed onto the surfaces of sediments in lakes or rivers (Brown and Laboureur, 1987).

Many dyes, especially azo dyes, are found to be degradable under anaerobic conditions (Brown and Laboureur, 1987, Brown and Hamburger, 1987, Gardner, 1990, Holdsworth et al., 1990, Loyd, 1992), such as those found in a sludge digester or anaerobic sediments in the aquatic environment. Microbes are able to biochemically cleave the azo bond and form aromatic amines, which are not readily biodegradable under anaerobic conditions (Brown and Laboureur, 1987), but are generally biodegradable under aerobic conditions (ETAD, 1986). Many aromatic amines have been shown to be carcinogenic, thus their formation by anaerobic degradation of dyestuffs is a major environmental concern (Singer and Grunberger, 1983).

Analytical Chemistry of Dyes

The possible environmental hazards presented by dyes make important the development of reliable monitoring methods for these compounds and their degradation products. Standard methods for their analysis do not exist, and their great

variety impede the development of such. Various researchers have experimented with different ways of separating, detecting, and identifying dyestuffs and related chemicals, most involving some sort of liquid chromatography and/or mass spectrometry. As previously mentioned, dyes are challenging to analysts because of their size, polarity, and thermal lability.

Liquid Chromatography

Most environmental samples occur as mixtures of compounds. *Chromatography* is a technique for separating the compounds in a mixture. In chromatography, a gaseous or liquid mobile phase carries the sample through a column containing a liquid or solid stationary phase. Each compound in the mixture partitions itself between the stationary and mobile phases. Since each compound spends the same amount of time in the mobile phase but (hopefully) different amounts of time in the stationary phase, the compounds will elute from the end of the column at different times and thus may be detected in turn.

Many organic compounds of environmental concern are separated and analyzed by gas chromatography (GC), in which a sample is volatilized by heat and passed through a column in the gas phase. Unfortunately, most dyes cannot be analyzed

by GC because they are too large and polar; they are destroyed by heat before they volatilize. For dyes and other such compounds, separation is best performed by liquid chromatography (LC), in which the mobile phase is a liquid such as water, methanol, acetonitrile, or hexane, and the stationary phase is solid. Most liquid chromatography of dyes has been performed with *reverse phase* LC, involving a polar mobile phase and non-polar stationary phase. The term HPLC refers to LC performed at high pressures.

The polarity of ionic organic compounds, such as sulfonated azo dyes, may limit retention on non-polar stationary phases, and thus limit resolution. A special type of reverse phase LC which helps ameliorate this problem is *ion-pair chromatography* (IPC), in which a counterion is added to the mobile phase to improve retention of the analyte. There are two theories of how IPC works. The ion-pair reagent may form a neutral ion-pair in solution with the analyte which behaves as a nonionic analyte. Alternately, the ion-pair reagent may sorb to the mobile phase and retard the analyte through an ion-exchange mechanism (Miller, 1988). Common ion-pair reagents used to separate acidic compounds are tetrabutylammonium hydroxide, tetraethylammonium hydroxide, and hexadecyltrimethylammonium bromide (Miller, 1988).

UV/Visible Detection: LC uses a variety of detectors, including UV/Vis, fluorescence, amperometric, refractive index, and mass spectrometry (Miller, 1988). Since dyes absorb strongly at visible and ultraviolet wavelengths, UV/Vis is often the detection method of choice when mass spectrometric information is not needed. By setting the detection wavelength at the maximum wavelength of absorption for a dye, sensitivity can be maximized and interference from other compounds minimized. A special kind of UV/Vis detector is the diode array detector (DAD) which allows not only the generation of chromatograms by detection at several wavelengths, but can also give the entire UV/Vis absorption spectrum for a given peak between 200 nm and 600 nm. This can give limited structural information about the dye and helps confirm the identity of a given peak.

Tincher (1986) was able to detect acid dyes in river water samples at the parts-per-billion level by using a resin-adsorption procedure followed by LC/UV-Vis. Two liters of sample were passed through Amberlite XAD-2 resin columns, and the dyes were extracted from the resin with 250 mL of pyridine, which was evaporated to dryness. The solid left was dissolved in 10 mL of methanol and dimethylformamide, concentrating the dye by 200 times from the original sample.

The samples was then separated on a Spherisorb ODS analytical column using gradient elution of water, methanol, and the ion-pair reagent tetrabutylammonium hydroxide.

Fischer et al. (1990) separated and identified several natural and synthetic textile dyes using LC/DAD with gradient elution of acetonitrile and a phosphate buffer in water. Analytes included anthraquinones, flavones, flavonoles, and sulfonated azo dyes, separated on a Lichrosorb RP 8 analytical column. The researchers found that most of the compounds could be easily distinguished by retention time, UV-Vis spectrum, or both. Compounds with similar retention times and spectra could be distinguished by comparing their ratios of absorbance at two wavelengths. Identification of compounds was performed by comparison with standards. Retention times of the sulfonated azo dyes varied greatly with the pH of the phosphate buffer; they decreased with increasing pH. However, many dyes exhibited tailing peaks at very low pH, so a pH of 3.5 was preferred.

Giolando et al. (1990) performed LC/DAD separation/detection of sulfonated azo dyes (acid dyes) on a reverse phase octadecasilyl analytical column using linear gradient elution of water and acetonitrile. It was desired to detect dyes at trace levels in samples from an activated sludge

basin. For this purpose, the samples were concentrated by solid phase extraction onto an XAD-2 resin and subsequent Soxhlet extraction into acetonitrile. This allowed detection of azo dyes at the part-per-billion level.

White and Catterick (1990) used LC/DAD to separate two acidic monoazo dyes and calculate peak purity parameter (PPP) values and complementary chromaticity coordinate (CCC) values. Chromatography was performed on a polymeric packing material with isocratic elution of acetonitrile, water, and the ion-pair reagent tetrabutylammonium hydrogen sulfate. PPP values are the mean wavelengths of a spectrum weighted by absorbance. CCC values are a qualitative color measurement system based on a mathematical analog of the human eye. Both PPP and CCC values are specific to individual dyes and thus can reduce large amounts of spectral data to just a few numerical values. White and Catterick found that the use of PPP and CCC values improved the qualitative analysis of dyes.

Electrochemical Detection: Sturrock (1987) and Camp (1989) were able to separate and detect direct dyes, acid dyes, and substituted anilines at low parts-per-billion levels using isocratic reverse phase chromatography and a novel swept-potential voltametric detector. This instrument monitors the current generated by the flow of electrons from

an electrode to analytes which are being reduced in the detection cell. In addition to generating a chromatogram by detection of current produced at a single voltage (potential), the detector measures and plots current versus voltage over a one volt range every second. Since different compounds have different reduction potentials, this detector gives data which can help identify chromatographic peaks. Azo dyes responded well to this detection method due to their ability to be reductively cleaved to aromatic amines.

Liquid Chromatography/Mass Spectrometry

Mass spectrometry (MS) is a powerful analytical technique used to obtain information about the molecular weight and structure of an unknown compound. In mass spectrometry, analyte molecules are ionized and sometimes fragmented by some transfer of energy (Watson, 1985). The molecular ion or fragment ions are detected according to their mass divided by their charge, or m/z value. In principle, the molecular weight and fragmentation pattern of a compound may be used to identify it uniquely. Mass spectrometers are very useful for environmental analysis because they are capable of detecting only a few nanomoles of sample (Watson, 1985), and they can be interfaced with gas and liquid chromatographs.

Once again, dyes present a challenge to mass

spectroscopists because of their polarity and thermal lability. Conventional ionization techniques, such as electron impact (EI) or chemical ionization (CI), require analytes molecules to migrate by diffusion from the inlet to the ionization chamber (Watson, 1985). The extremely low vapor pressures of large dyes, especially sulfonated dyes, prevents this migration. In addition, dyes do not form thermally stable derivatives required by these ionization techniques (Yergey et al., 1990).

Besides the problem of getting nonvolatiles into the gas phase is the problem of interfacing a liquid chromatograph with a mass spectrometer; a packed column LC/MS must handle molecular flow rates up three orders of magnitude greater than a capillary GC (Yergey, 1990). A high pressure flow (around 300 psi) from the LC must be converted to a vapor compatible with the extremely low working pressures of mass spectrometers of about 10^{-5} torr (Budde et al., 1990). The last two decades have seen the development of several effective LC/MS interfaces/nonvolatile ionization techniques, including particle beam (PB), thermospray (TSP), atmospheric pressure ionization (API), and electrospray (ES).

Thermospray LC/MS

In thermospray LC/MS, flow from the LC is passed through

a heated capillary tubing which nebulizes and partially vaporizes the liquid. Liquid which is not vaporized enters the ion source through a probe as a fine aerosol spray, and ionization of nonvolatiles occurs by direct ion evaporation of the highly charged liquid particles (Yergey, 1990). Thermospray is considered a "soft" ionization technique, as it generally produces little fragmentation. An electron gun may also be used to effect chemical ionization for more volatile compounds. A small portion of the vapor/mist diffuses into the mass analyzer which can be either a quadrupole or magnetic sector instrument. It is critical in thermospray to control the vaporizer temperature so that complete thermal vaporization does not occur (Yergey, 1990).

Voyksner (1985) characterized nonsulfonated azo and anthraquinone dyes in environmental samples using thermospray LC/MS. Separation of azo dyes was performed on a Zorbax C18 column using gradient elution of methanol and 0.1 M ammonium acetate. In positive-ion mode, thermospray analysis produced mostly $[M + H]^+$ ions with little fragmentation. Similarly, in the negative-ion mode $[M]^-$ ions were produced with little fragmentation. Fragmentation, and thus structural information, was enhanced by the activation of a filament at the tip of the TSP vaporizer which was operated at 200 eV.

TSP LC/MS proved to be a very effective way to analyze for azo dyes in wastewater, which it could detect down to 10 parts-per trillion.

Ballard and Betowski (1986) used thermospray ionization/tandem mass spectrometry without chromatographic separation to analyze sixteen commercial dyes and three liquid dye-manufacturing wastes, including seven nonsulfonated azo dyes and one sulfonated azo dye. Collision-activated dissociation (CAD) was used to generate daughter ions for MS/MS; in this technique argon was used as a collision gas with a collision energy of 20 eV. In the positive-ion mode, detection limits ranged from 20-200 ng, and were about one order of magnitude higher in the negative-ion mode. Daughter ions of azo dyes were formed by cleavage of the azo bond.

Flory et al. (1987) obtained LC/negative-ion thermospray mass spectra for azo dyes with up to four sulfonic acid groups. Neutral pH gave the best results, and low nanogram detection limits were obtained. Both singly and doubly charged ions were detected due to the loss of one and two sodiums, respectively. In one case a triply charged ion was observed due to dimerization and the loss of three sodiums. The researchers found that response could be optimized with respect to ammonium acetate concentration in the LC mobile

phase.

Yinon et al. (1988) performed thermospray ionization, without chromatography, on a series of dyes including sulfonated azo dyes. Very low detection limits (0.05 - 20 ng) were obtained by adding a wire repeller to the ion source. Positive-ion mass spectra of several acid dyes were obtained for the first time due to the enhanced sensitivity of the system provided by the repeller. The loss of Na, 2 Na's, SO₃Na, and 2 SO₃Na's, and replacement by hydrogen was observed.

Similarly, McLean and Freas (1989) modified a thermospray vaporizer probe and ion source to enhance LC/MS analysis of five disulfonated azo dyes. Separation was performed on a Spherisorb ODS II reverse phase column with gradient elution of ammonium acetate and acetonitrile. The thermospray vaporizer probe tip orifice was restricted, which enhanced sensitivity by decreasing droplet size in the aerosol spray and thus increasing the charge-per-unit area of the droplets. A needle repeller added to the ion source increased sensitivity by causing greater extraction of ions into the mass analyzer.

Yinon et al. (1989) also used a wire repeller to enhance thermospray LC/MS of a disperse azo dye. With maximum ion intensity occurring at a repeller voltage range of 200 - 275

V, the researchers were able to obtain a minimum detection limit of 10 ng; this is approximately two orders of magnitude more sensitive than analysis with a repeller voltage of 0 V. It was believed that the electric field between the repeller and the entrance funnel to the mass analyzer accelerated ions toward the funnel and into the mass analyzer.

Particle Beam LC/MS

A disadvantage of thermospray is the relatively high operating pressure (0.1 - 1 torr) of the aerosol spray, which causes instabilities in ion intensities and limits the generation of gas-phase ions (Budde et al., 1990). Also, because thermospray is a "soft" ionization technique, it often fails to provide the structural information necessary to identify an unknown. An alternate LC/MS interface developed to solve this problem is the particle-beam (PB) interface, in which an aerosol spray is generated not by heat but by mixing of the liquid flow with a non-reactive gas such as helium. The spray is carried by the gas into a heated chamber where the aerosol particles are reduced in size by desolvation (Budde et al., 1990), and then accelerated into the ion source through a two-stage pressure reducer. Most of the vapor is removed by collision with skimmers, but particles with the correct momentum are carried in a tight beam to an ionization

source such as electron impact or chemical ionization. Ions are also formed by direct ion evaporation as in thermospray.

Yinon et al. (1989) used a particle beam interface, electron impact ionization, and quadrupole mass analyzer to perform LC/MS of a series of commercial dyes including nonsulfonated azo dyes. Dyes were separated from impurities on a Varian MicroPak MCH-5-N-CAP C18 column by gradient elution of methanol and water. The system was found to be two to three orders of magnitude less sensitive than thermospray with wire repeller. However, good EI fragmentation patterns were achieved. Fragmentation occurred by cleavage of the azo bonds and addition of hydrogen to form amines, and cleavage of carbon-nitrogen bonds adjacent to the azo bonds.

Behymer et al. (1990) utilized LC/particle beam/EI-MS for the analysis of several compounds of environmental interest, including three aromatic diamines. Separation was performed by gradient elution of methanol and 0.01 M ammonium acetate on a C18 packed analytical column. The researchers found that precision, linear calibration, and detection limits (10 to 440 ng) suggest that this type of system can provide quantitative analyses of nonvolatiles.

Atmospheric Pressure Ionization LC/MS

With LC/API/MS, flow from the LC is pneumatically

nebulized and thermally desolvated at atmospheric pressure, and ionization is initiated by low-energy electrons from a corona discharge (Watson, 1985) which generate ions by gas-phase chemical ionization (Bruins et al., 1987). Ions are then introduced to the mass analyzer through pinhole skimmers which maintain the low pressures necessary in the mass spectrometer. Bruins et al. (1987) utilized an atmospheric pressure ionization (API) interface for the LC/MS/MS analysis of several acid dyes. Chromatography was performed on a Altex Ultrasphere C18 column with a mobile phase of acetonitrile and 0.1 M ammonium acetate. The researchers reported that the sulfonated dyes produced strong negative-ion mass spectra, and that collision-induced dissociation of parent ion scans show SO_3^- as a characteristic fragment.

Desorption Ionization Techniques

The first methods to obtain useful mass spectra of large, nonvolatile molecules were desorption ionization methods. These techniques, such as fast atom bombardment (FAB), and secondary-ion mass spectrometry (SIMS) involve analysis of the sample in the condensed phase, either solid or dissolved in a viscous solvent (Watson, 1985). Analyte molecules are ionized by bombardment of high-energy particles. It should be noted that desorption ionization methods are not readily

interfaceable with liquid chromatographs, though research is being done in this area.

With FAB, a sample is dissolved in a viscous solvent such as glycerol and mounted on a probe. The sample is then subject to bombardment by a 6 - 10 keV beam of atoms, usually argon or xenon, that disrupts particles from the surface of the sample by a violent mechanism called "sputtering". Ions are preformed in solution and also form during flight to the mass analyzer; most ions detected are adducts of the analyte, the matrix, and salts such as sodium and potassium. SIMS is very similar to FAB and may operate by the same principle, but uses a beam of primary ions such as Ar^+ or Xe^+ (Watson, 1985). Several researchers have used FAB or SIMS to analyze dyestuffs.

Monaghan et al. (1982) was able to obtain mass spectra for seven sulfonated azo dyes using FAB-MS. The dyes were dissolved in glycerol and bombarded by a 6 keV argon beam. Negative-ion mode gave stronger signals than positive-ion mode and mass spectra were obtained for dyes with up to five sulfonate groups, though the signal grew poorer as the number of sulfonate groups increased. All dyes showed $[\text{M} + \text{Na}]^+$ and $[\text{M} - \text{Na}]^-$ ions in the positive- and negative-ion modes, respectively. Fragmentation occurred by cleavage of the azo

bond, but only on sulfonated parts of the dyes.

Shimanskas et al. (1989) have described a simple method for enhancing the spectral quality of FAB mass spectra of sulfonated dyes, involving passing the commercial dye through an Amberlite IR-118-H synthetic ion-exchange resin. This removes inorganic salts which can suppress ionization of the dye, and converts the sodium-sulfonate groups on the dye to free acids. Treated dye gives stronger signals and less confusing adduct formation than untreated samples.

Richardson et al. (1992) used liquid SIMS/MS/MS to analyze monosulfonated and disulfonated azo dyes. Tandem MS was used to gain structural information which otherwise may be lost due to matrix interferences. Primary ions were obtained from a cesium gun, and the SIMS matrix was 3-nitrobenzyl alcohol. The researchers reported that many structurally characteristic ions were obtained. Fragmentation occurred by cleavage of the azo bond and of carbon-nitrogen bonds adjacent to azo bonds, on sulfonated and unsulfonated parts of the molecule. Other fragments ions were observed due to the loss of SO_2 , SO_3 , NO_2 , and NHCOCH_3 .

CHAPTER III

METHODS AND MATERIALS

This research consisted largely of developing methods for the qualitative and quantitative analysis of several organic dye compounds in aqueous samples; specifically, three sulfonated azo dyes and their degradation products. It was also desired to confirm the known structures of the hydrolyzed dyes (Remazol Black B and Remazol Gold Yellow 3RA) and identify the structure of Remazol Red RB. Achieving this goal required the use of several types of analytical instrumentation and methods of sample preparation.

Experiments performed fell into three basic categories. The first was the development of liquid chromatographic methods for the resolution and detection of dyes and their reduction products. This was performed with aqueous dye solutions prepared from powdered dye from the manufacturer, and such solutions were reduced by the addition of sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$). The second category was the mass spectrometric identification of the structures of the dyes and their reduction products. This was performed with both gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) techniques. The

size and polarity of the sulfonated azo dyes and many of their reduction products precluded the use of GC/MS and made LC/MS a special challenge. The third category was the application of these methods to actual wastewater samples, both treated and untreated.

Types of Samples

Parent Dyes from Manufacturer

The industrial wastewater of interest was the Navy 106 Dye Washwater from a textile dyeing plant in Martinsville, Virginia, which contained three major sulfonated azo dyes: Remazol Black B (70%), Remazol Red RB (26%), and Remazol Gold Yellow 3RA (4%). All three dyes were obtained from the manufacturer, Hoechst-Celanese Corp., as the stable sulfatoethyl sulfone (parent) form. The parent dyes were solids cut with approximately 55% sodium chloride and contained several other impurities, probably reagents used in dye synthesis.

Vinyl Sulfone and Hydrolyzed Dyes

As mentioned previously, the dyes containing the Remazol reactive group are stored and transported in a stable sulfatoethyl sulfone form, but react at high pH to form a

vinyl sulfone group which either reacts with cotton or undergoes a competing hydrolysis reaction. In order to distinguish between the parent, vinyl sulfone, hydrolyzed species of each dye on the HPLC, it was necessary to prepare aqueous solutions of each form for each Remazol dye. To convert the sulfatoethyl sulfone groups to their reactive vinyl sulfone forms, aqueous solutions of each parent dye were brought to pH 11.0 with 1 N sodium hydroxide, stirred for an hour at room temperature, then brought to neutral pH with 1 N hydrochloric or sulfuric acid. To hydrolyze the vinyl sulfone form of the dye, the solution was kept at pH 11.0, refluxed for three hours, and then brought to neutral pH with 1 N hydrochloric or sulfuric acid (Weber, 1990).

Reduced Dyes

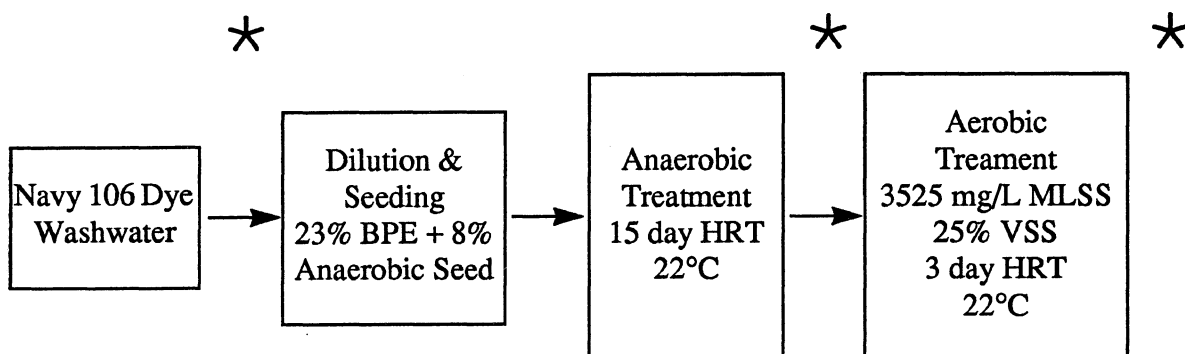
Also analyzed were chemically reduced dye solutions. Sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), at a concentration of 500 - 1000 mg/L per 100 mg/L of dye in solution, was added to aqueous solutions of parent, vinyl sulfone, and hydrolyzed Remazol Black B, Remazol Red RB, or Remazol Gold Yellow 3RA. The reaction was performed at pH 6.5 - 7.5 and usually took less than five minutes for all of the original dye color to disappear. In addition, experiments were performed in which less than stoichiometric amounts of reducing agent were added

incrementally to dye solutions, to form intermediate products of dye reduction.

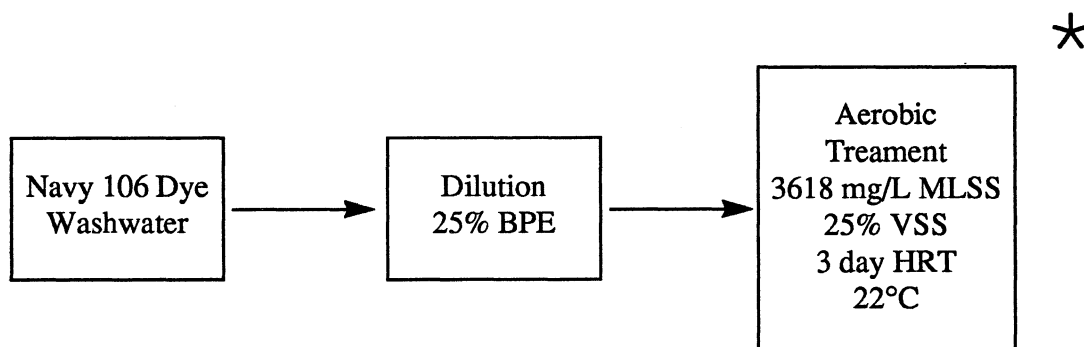
Wastewater Samples

Navy 106 Dye Washwater was obtained from the pad dyeing process of a textile plant in Martinsville, Virginia. Samples of untreated Navy 106, anaerobically treated Navy 106, and anaerobically/aerobically treated Navy 106 Washwater were analyzed.

Biologically treated washwater samples were obtained from two experimental treatment processes of Navy 106 set up and described by Loyd (1992), and shown schematically in Figure 11. A mixture of 69% Navy 106, 23% Blacksburg-VPI Sanitation primary effluent (BPE) (which consisted primarily of domestic wastewater), and 8% anaerobic seed was sealed in 26 L Nalgene carboys at room temperature with no agitation for 15 days at 22 degrees C. Following this anaerobic treatment, the mixture was treated aerobically in two parallel 9.7 L Continuous Flow Biological Reactors (CFBRs) with a hydraulic retention time of 3 days and an MLSS of about 3500 mg/L. Aeration and agitation was provided by an air stone. Samples for LC/DAD and GC/MS analysis were taken before treatment, after anaerobic treatment, and after anaerobic and aerobic treatment.



Anaerobic/Aerobic Treatment Scheme



Aerobic Treatment Scheme

★ indicates point in process where samples were taken for LC/DAD and GC/MS analysis

Figure 11: Treatment schemes of Navy 106 Dye Washwater used by Loyd (1992), and stages of the processes where samples were taken for analysis by liquid chromatography/diode array detection (LC/DAD) and gas chromatography/mass spectrometry (GC/MS). BPE = Blacksburg primary effluent; HRT = hydraulic retention time; MLSS = mixed liquor suspended solids; VSS = volatile suspended solids (from Loyd, 1992).

The TOC of untreated Navy 106 Washwater was between 289 mg/L and 380, and it generally had a pH between 10.5 and 12. Loyd found that the anaerobic treatment scheme described caused a 90% reduction in American Dye Manufacturers Institute (ADMI) color value of the Navy 106, going from 9584 ADMI to 5223 ADMI after dilution with Blacksburg primary effluent and anaerobic seed, and down to 980 ADMI after anaerobic treatment. However, anaerobic treatment caused only a four percent decrease in TOC of the Navy 106. Loyd indicated that subsequent aerobic treatment actually led to a statistically significant 2% increase in ADMI color value, but reduced TOC by 47% from the original, not including the effect of dilution. Anaerobic/aerobic treatment significantly reduced the toxicity of Navy 106, resulting in an LD₅₀ of 56% in tests on *Ceriodaphnia dubia*.

Standard Preparation

Sample purification

To prepare a standard for quantitative analysis of Remazol Black B and Remazol Red RB, it was necessary to purify the solid dye as received from the manufacturer. Because the dyes themselves are sulfonated and therefore anionic, they

behave very similarly to the salts from which they must be separated, making purification difficult. Several methods of purification were attempted.

Alumina Column Chromatography: One method employed for separating Remazol Black B and Remazol Red RB from inorganic salts and other impurities was alumina open-column chromatography. In this method, a 20 mm x 20 cm glass column was slurry-packed with 150 mesh neutral alumina (Al_2O_3) in a solution of 50% water and 50% methanol, and overlain by a fine quartz sand. A small volume of concentrated dye in water and methanol was placed at the top of the column and gravity washed through the column. The ratio of water to methanol in the mobile was increased by 5% after each empty column volume of mobile phase passed through the column. The colored band of purified dye was collected as it eluted, then concentrated as described below.

HPLC Fraction Collection: Purification of Remazol Black B and Remazol Red RB was also performed by the collection of HPLC fractions. Chromatography was performed on a Hewlett-Packard 1090 Liquid Chromatograph with a preparatory scale (250 mm x 7 mm) Econosphere C18 5 μm column. Up to 0.25 mL of concentrated dye was injected onto the column, and eluted isocratically with a flow of 2 mL/min. and a mobile phase

composition of 60% water / 40% methanol. Pure dye peaks were collected manually from multiple injections of dyes, then freeze-dried to solid as described below.

Sample Concentration

The dye purification methods described above resulted in relatively dilute solutions of pure dye (1 - 10 mg/L). To obtain solid dye for standard preparation, and also to improve HPLC detection of dyes and their reduction products in other samples, it was necessary to concentrate dye solutions and wastewater samples. This was attempted by two methods: solid phase extraction and freeze-drying.

Solid Phase Extraction: Solid phase extraction of dye solutions was performed on the Bond Elut / Vac Elut system, made by Analytichem International (Harbor City, CA). The system consists of a vacuum basin and cover containing ten luer fittings, into which Bond Elut columns are placed. Unused fittings are covered with plugs. The Bond Elut columns contained either one cc or three cc's of sorbent material. Extraction was attempted with three different sorbent materials: octadecylsilyl, cyanopropyl, aminopropyl, and a proprietary strong anion exchanger (SAX). The columns were first conditioned with by rinsing with one column volume of methanol and one column volume of buffer at pH 7.0. The

aqueous dye sample was adjusted to pH 7.0 with 0.1 M HCl or 0.1 M NaOH. Sample volumes of 500 mL were passed through each Bond Elut column. Products were eluted from the column with one to five ml of 1 N HCl. Percent recovery of original Remazol Black B in solution was obtained by comparing absorbance of the original solution at 590 nm on a Beckman (Fullerton, CA) DU-6 Spectrophotometer, to that of the recovered extraction, and adjusting for dilution to find the recovered mass.

Freeze-Drying: After HPLC peaks were collected, they were placed in 12 mL Nalgene high speed polysulfone centrifuge tubes and freeze dried in SpeedVac Concentrator 100H coupled to a Refrigerated Condensation Trap 4104, both manufactured by Savant Corporation. A portion of the solid dye thus obtained was analyzed by LC/DAD to verify purity.

Liquid-liquid Extraction: In preparation for analysis by GC/MS, liquid-liquid extraction of base/neutral and acid extractables was performed according to E. P. A. Method 625 (E. P. A., 1982). 100 µg/L of the following surrogate standards were added to aqueous samples as a quality control check:

Base/Neutral Fraction: Nitrobenzene-d₅, p-Terphenyl-d₁₄,
2-Fluorobiphenyl

Acid fraction: Phenol-d₆, 2-Fluorophenol, 2,4,6-Tribromophenol

One liter of aqueous sample was adjusted to pH > 11 by 10 N NaOH and placed in a two liter separatory funnel. A volume of 60 mL methylene chloride was added and the funnel was shaken for two minutes with periodic venting. After the organic layer separated, it was collected in a 250 mL flask. Two additional extractions into 60 mL methylene chloride were performed. The aqueous samples was then adjusted to pH < 2 by H₂SO₄ and three additional 60 mL methylene chloride extractions were performed. Each 180-mL fraction collected was placed in a Kuderna-Danish (K-D) concentrator consisting of a 10-mL concentrator tube connected to a 500-mL evaporative flask attached to a three ball Snyder column. The K-D apparatus was placed in a 60-65 degree water bath and each extract was concentrated to a volume of one mL.

Liquid Chromatography/Diode Array Detection

Liquid chromatographic analysis was performed on a Hewlett-Packard 1090 Liquid Chromatograph with Diode Array Detection (DAD). The DAD is equipped with a deuterium lamp which generated several chromatograms of a sample

simultaneously by detection of absorbance at several wavelengths. In addition, it scanned each chromatographic peak for its UV/Vis spectrum between 200 nm and 600 nm. For monitoring azo dyes and their degradation products, the DAD was set to detect absorbance at 254 nm, 450 nm, and 590 nm. To ensure proper working order of the instrument and monitor the strength of the deuterium lamp over time, Alltech's Reverse Phase Test-Mix D was run first each day of LC analysis. Instrument noise was defined as twice the average variation in the baseline of a typical 40 minute analysis (Miller, 1988), measured over at least nine analyses.

Chromatography was attempted on three different columns: Alltech C18, Zorbax Rx-C18, and Dionex Omnipac PAX-500.

Alltech C18

Reverse phase chromatography was performed using a 4.6 mm x 25 cm C18 column manufactured by Alltech Associates, Inc. (Deerfield, IL), with C18 guard column. Two gradient elution programs were developed, called the C18 reverse phase and the C18 ion-pair programs. The C18 reverse phase program used a mobile phase which consisted of methanol and 20 mmol acetate buffer, prepared by mixing 20 mmol acetic acid and 20 mmol ammonium acetate until pH 5.0 was achieved. The gradient concentrations are presented in Table 1.

Table 1: C18 reverse phase gradient for LC analysis of sulfonated azo dyes

Time (min.)	% 20 mmol pH 5.0 Acetate Buffer	%Methanol
0.0	80.0	20.0
1.0	80.0	20.0
15.0	0.0	100.0
20.0	80.0	20.0

To improve separation of polar reduction products, the C18 ion-pair program was developed. The mobile phase consisted of a pH 5.0, 20 mmol acetate buffer, methanol, and 20 mmol tetrabutylammonium hydroxide in water adjusted to pH 7.0 with glacial acetic acid as the ion-pair reagent. The conditions presented in Table 2 describe the ion-pair gradient.

Zorbax Rx-C18 Column

Many azo dye reduction products were expected to be amines which are basic in nature. Therefore, it was desired to use an LC column which had a base-deactivated stationary phase to prevent peak-tailing and irreversible adsorption of basic compounds into the column. The Zorbax Rx-C18, 4.6 mm I.D. x 25 cm, contains octadecyl material bonded to a silica support which is designed to prevent adsorption of basic compounds, and is manufactured by Mac-Mod Analytical, Inc. (Chadds Ford, PA). The column was used with the C18 reverse phase gradient described above.

Omnipac PAX-500 Column

The Omnipac PAX-500 Analytical Column and accompanying Omnipac PAX-500 Guard Column, made by Dionex Corporation (Sunnyvale, CA), consisted of an ethylvinylbenzene/divinylbenzene polymeric core coated with a polymeric colloid

Table 2: C18 ion-pair gradient for LC analysis of sulfonated azo dyes and their reduction products

Time (min.)	%20 mmol TBAH	% 20 mmol pH 5.0 Acetate Buffer	%Methanol
0.0	45.0	25.0	30.0
5.0	45.0	25.0	30.0
15.0	10.0	50.0	40.0
30.0	40.0	0.0	60.0
35.0	40.0	0.0	60.0
40.0	45.0	25.0	30.0

TBAH = Tetrabutylammonium hydroxide

which had been functionalized with a quaternary ammonium base. The column may be used simultaneously for both reverse phase and anion exchange chromatography. Sulfonated azo dyes and their sulfonated degradation products are organic anions, and thus the Omnipac PAX-500 column was utilized in an attempt to improve resolution of these compounds. Several mobile phase gradients were attempted, using water, acetonitrile, and a third eluant consisting of 1 M NaCl and 4 mM NaOH.

Quantitation of Dyes

Standard curves were prepared from purified, hydrolyzed Remazol Black B and Remazol Red RB. Stock solutions of these dyes were prepared at concentrations of 1, 5, 10, 25, 50, and 100 mg/L dye, then analyzed by LC/DAD. By performing a linear regression on the plot of peak area versus concentration, a straight line was obtained with slope equal to the mass absorbtivity coefficient times the path length.

Liquid Chromatography/Mass Spectrometry

LC/MS analysis was performed by Dr. Robert D. Voyksner of the Analytical and Chemical Services of Research Triangle Institute, Research Triangle Park, North Carolina. LC/MS was performed on a Hewlett-Packard (Palo Alto, CA) 5989 Mass

Spectrometer coupled to an Analytica-Branford (Branford, CT) Electrospray LC/MS interface. Both positive-ion and negative-ion electrospray analyses were performed. Operating mass range was 50 - 1000 daltons, and typical voltage was 250 eV or - 250 eV. Dye concentrations in samples analyzed by LC/MS were about 0.1 µg/uL. Analysis was performed off-line, with LC fractions collected from gradient programs described above. Data was acquired and outputted manually.

Gas Chromatography/Mass Spectrometry

All GC/MS analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph with a 30 m x 0.25 mm ID DB-5 capillary column and a Hewlett-Packard 5790 Mass Selective Detector data system. The electron energy was 70 eV and the source pressure was 4×10^{-5} torr. The GC injector temperature was 260 degrees C, and initial oven temperature was 80° C held for one minute and then ramped 10 degrees C per minute to a final temperature of 300 degrees C, and held for four minutes. The transfer line was held at 280 degrees C. Mass spectra were not background enhanced and were tentatively identified by matching to Wiley/NBS library and by manually interpreting the spectra.

CHAPTER IV

RESULTS AND DISCUSSION

Dye Purification

The solid dye powder, as received from the manufacturer, contained about 45% weight per weight sodium chloride and other impurities such as dye precursors. The following are the results of the two techniques applied to separate the dye from these contaminants: LC fraction collection and neutral alumina chromatography.

LC Fraction Collection

The collection of dye peaks as they eluted from the HP 1090 LC provided an effective method of dye purification. Each injection of 0.250 mL of concentrated hydrolyzed dye, at a concentration of 30 - 50 mg/L, typically yielded 2 - 4 mL of pure dye in methanol/water solution at a final concentration of 2 - 3 mg/L. Figure 12 shows a LC chromatogram of collected and combined fractions of hydrolyzed Remazol Black B; a single chromatographic peak indicated that the Remazol Black B was pure.

Alumina Column Chromatography

Neutral alumina open column chromatography failed to be

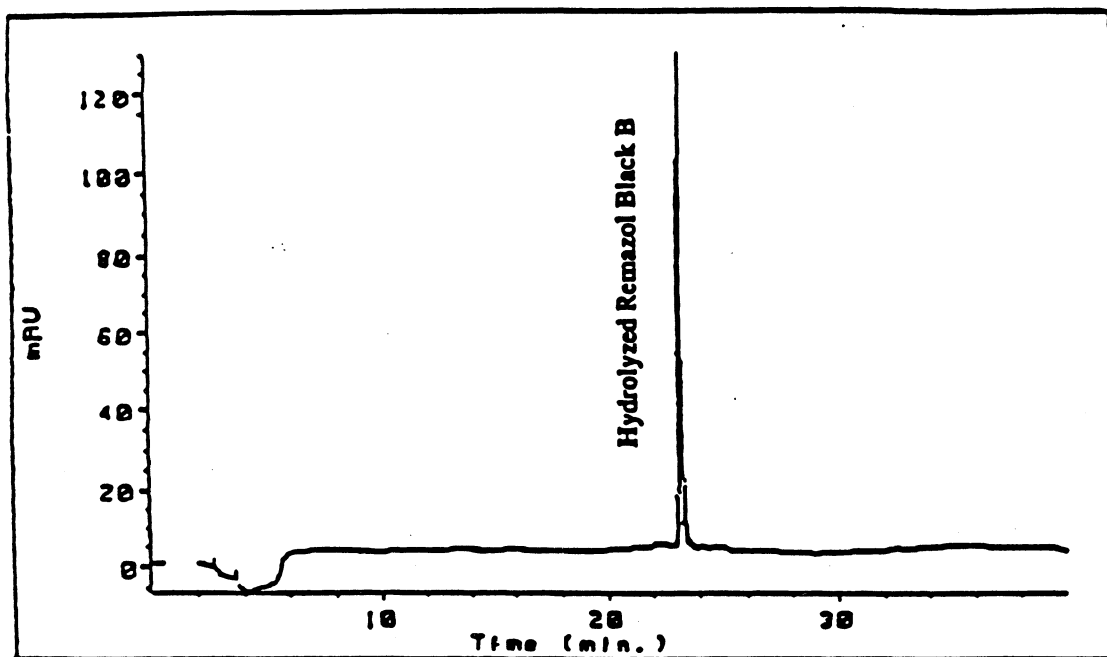


Figure 12: LC chromatogram of purified hydrolyzed Remazol Black B collected by LC fractionation and detected at 254 nm. Chromatograms generated by detection at 380 and 590 nm also showed a single peak. The single peak suggests high purity (mAU = milli-Absorbance units).

an effective method of dye purification. Ideally, a dye would travel down the column as a small colored band of material. In practice, the dye spread out over a large volume of the mobile phase/alumina as it moved down the column. For example, a 2 mL sample of 30 - 50 mg/L hydrolyzed Remazol Black B solution, placed at the top of the column, required collection of 100 - 150 mL of sample as the dye eluted from the bottom of the column. The band that eluted was very wide and uneven because dye moved at different rates over the cross section of the column, and possibly the dye reacted with the neutral alumina. LC/DAD analysis of the collected dye-colored sample revealed that non-dye contaminants still remained after attempted purification by neutral alumina. In addition, LC/DAD analysis of the column effluent indicated that Remazol Black B in the sulfatoethyl sulfone form was hydrolyzed as it passed through the column.

This method might be improved if the alumina were packed more uniformly over the volume of the column, or if suction was applied to the end of the column. Also, acidic or basic alumina may provide better chromatography than the neutral alumina which was used in this experiment. In any event, the limitations encountered with technique prevented further use for dye purification.

Sample Concentration

Solid Phase Extraction

As mentioned previously, four kinds of sorbent cartridges were used with the Bond Elut/Vac Elut system in an attempt to concentrate aqueous dye solutions: octadecyl, cyanopropyl, aminopropyl, and SAX materials. Of these four, only the SAX material, a quaternary amine strong anion exchanger, provided sufficient sorption of the dye onto the stationary phase to merit investigation of recovery upon elution. The other sorbents showed less than 10% retention of the dye.

After passing 500 mL of 1 mg/L hydrolyzed Remazol Black B in water through a 3 mL SAX cartridge, elution was attempted with 5 mL distilled water and also with 5 mL 1 N hydrochloric acid. Analysis of the extract by UV/Vis spectrophotometry at 590 nm indicated that approximately 2% of the original dye in solution could be recovered with 5 mL distilled water, and approximately 86% could be recovered with 5 mL 1 N hydrochloric acid. Thus, an aqueous solution of hydrolyzed Remazol Black B could be concentrated from 500 mL to 5 mL, a factor of 100, with about 86% recovery.

This technique could be used to concentrate dye in

aqueous solutions for the purpose of improving detection of dyes in dilute samples. However, quantification of dye in solution remained a problem due to the inability to predict sorption and recovery of the dye from wastewater samples which had varying ionic strengths. Because the dye must be eluted from the cartridges with a high ionic strength solution, this technique cannot be used in the preparation of a standard. Solids present in biologically digested samples also interfered with the effectiveness of solid phase extraction by physically clogging the cartridge. The most useful application would be for concentrating an aqueous sample in preparation for LC analysis for the presence of the dye(s) of interest.

Freeze-drying

Freeze-drying aqueous and aqueous/methanol solutions of dye provided an effective method of sample concentration. LC fractions collected in 12 mL polysulfone centrifuge tubes yielded solid dye when freeze-dried. LC analysis before and after freeze-drying revealed that no chemical alteration of dye occurred during this process.

Monitoring for Dyes with Liquid Chromatography

Aqueous solutions of Remazol Black B, Remazol Red RB, and Remazol Gold Yellow 3RA were prepared using solid dye from the manufacturer, and analyzed by LC/DAD. The variety of forms of each dye (sulfatoethyl sulfone, vinyl sulfone, and hydrolyzed) that potentially existed in samples required methods which could separate at least nine different compounds, plus near-isomers of the dyes which also existed in the dye solids. Three different columns (Alltech C18, Zorbax Rx-C18, and Omnipac PAX-500) were utilized with reverse phase and ion-pair chromatography to monitor for the dyes. UV/Vis spectra of the three dyes, which are very distinctive and allow identification of dye peaks on the LC/DAD, are shown in figures 13 - 15. Also shown are the structures of the sulfatoethylsulfone (parent) forms of Remazol Black B and Remazol Gold Yellow 3RA.

Alltech C18 Reverse Phase

The reverse phase LC method, performed on the Alltech C18 analytical column, provided a quick and effective method for detecting and quantifying sulfonated azo dyes in aqueous samples. The method was able to separate all three forms (parent, vinyl sulfone, and hydrolyzed) of Remazol Black B,

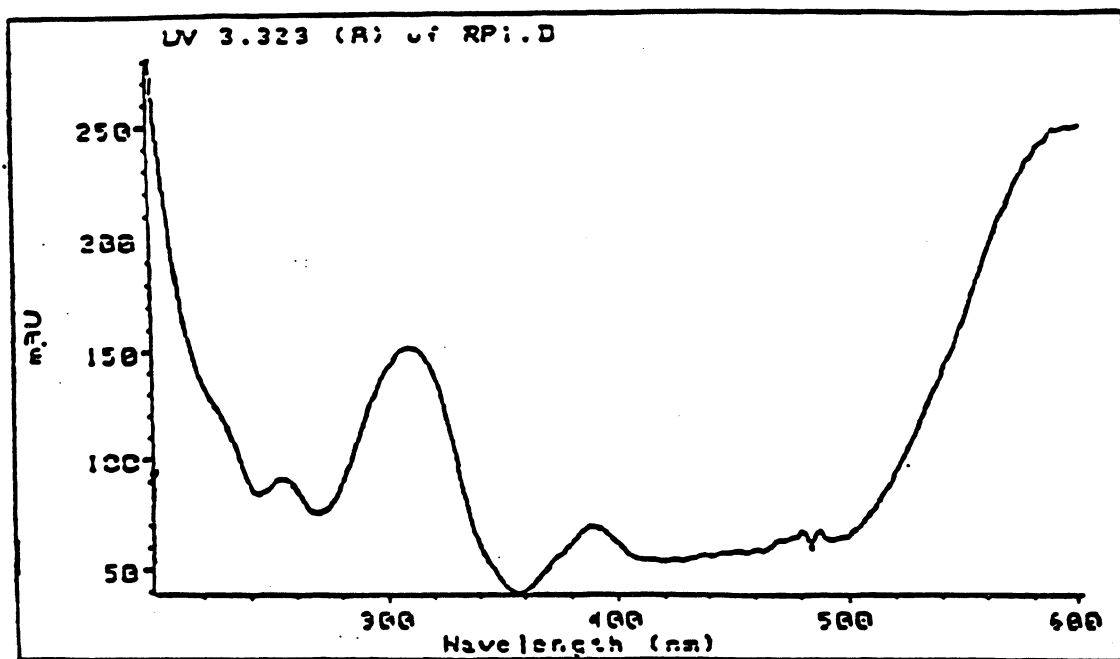


Figure 13: UV/Visible absorption spectrum of hydrolyzed Remazol Black B, as detected by diode array detector (DAD) after separation by liquid chromatography (LC) (mAU=milliabsorbance units).

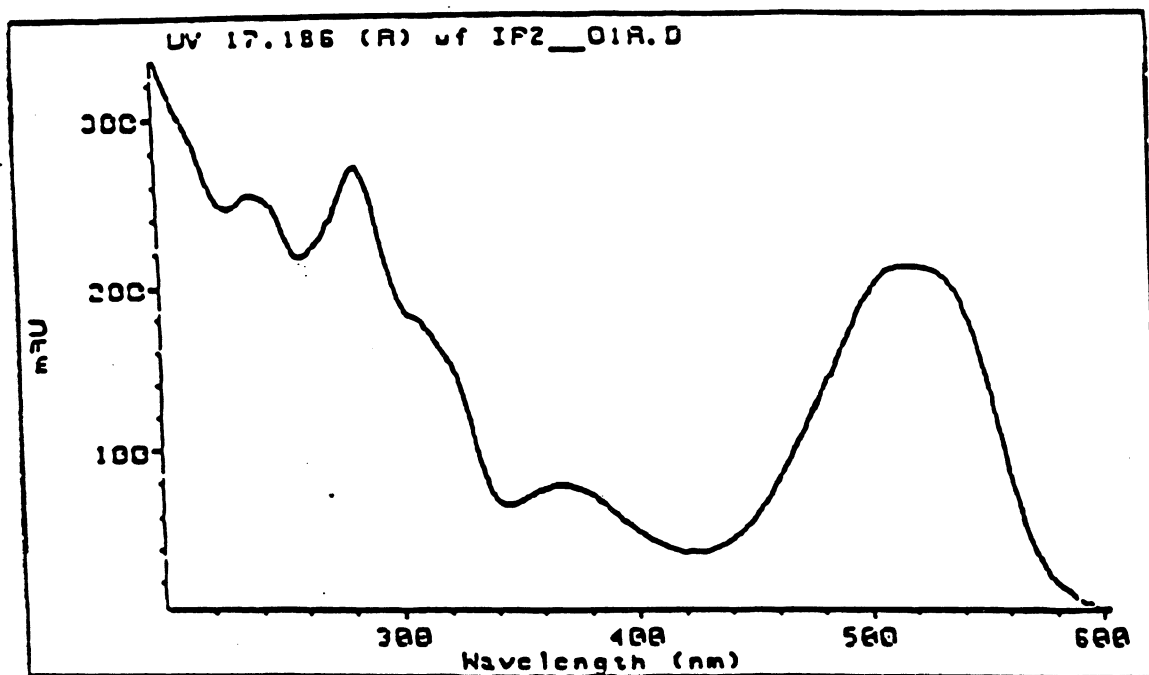


Figure 14: UV/Visible absorption spectrum of hydrolyzed Remazol Red RB, as detected by diode array detector (DAD) after separation by liquid chromatography (LC) (mAU=milliabsorbance units).

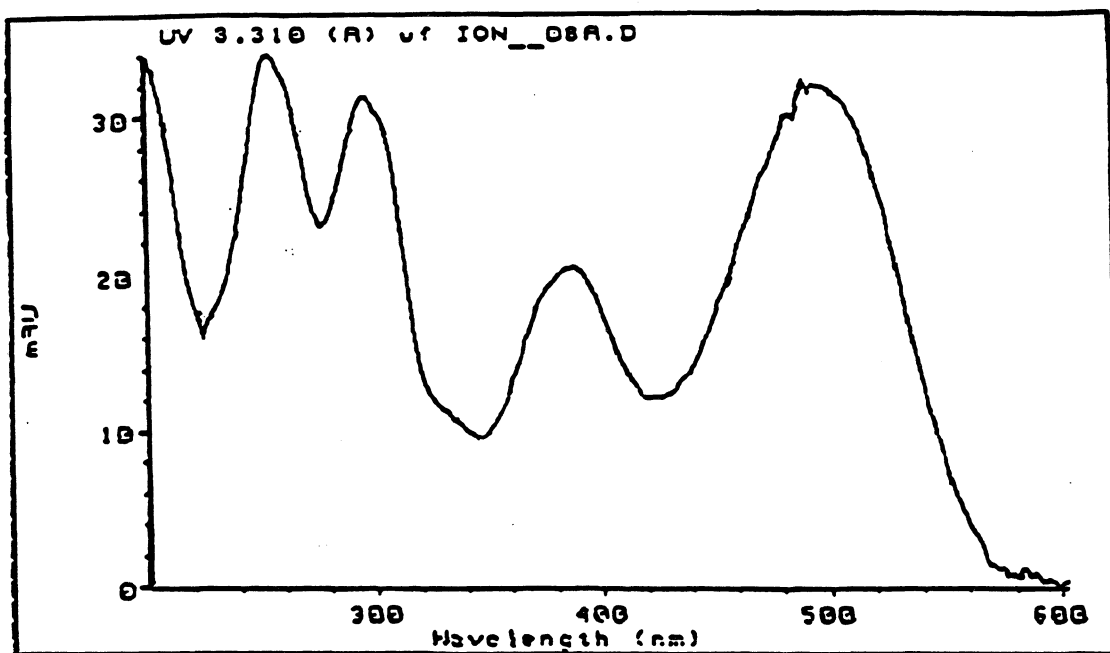


Figure 15: UV/Visible absorption spectrum of hydrolyzed Remazol Gold Yellow 3RA, as detected by diode array detector (DAD) after separation by liquid chromatography (LC) (mAU=milliabsorbance units).

Remazol Red RB, and Remazol Gold Yellow 3RA. Table 3 shows a listing of the retention times of the various forms of the dye. For each dye, the three forms eluted in the order expected from their respective polarities: parent form first, followed by hydrolyzed, and then vinyl sulfone.

Alltech C18 Ion-Pair

The ion-pair program with 20 mmol tetrabutylammonium hydroxide (TBAH) developed on the Alltech C18 column could also separate all three forms of the three dyes. Table 4 lists the retention times of these compounds as well as unidentified compounds in the dye powder. As the table indicates, retention times of the dyes were greatly increased with the ion-pair program as compared to the reverse phase program. Though decreasing the strength of the ion-pair reagent (tetrabutylammonium hydroxide) would decrease analysis time, this caused difficulty in the resolution of the hydrolyzed and vinyl sulfone forms of Remazol Black B. Besides the longer retention time, other disadvantages of the ion-pair method included tailing peaks, an unstable baseline relative to the C18 reverse phase method, and higher detection limits. However, as will be seen, the chief benefit of this method was the ability to resolve small, polar reduction products of the dyes.

Table 3: Average LC retention times of various forms of dye on the Alltech C18 analytical and C18 guard column with C18 reverse phase parameters

Analyte	Average retention time (min.)
Remazol Black B	
Parent	3.3
Vinyl sulfone	9.3
Hydrolyzed	8.2
Remazol Red RB	
Parent	7.4
Vinyl sulfone	8.7
Hydrolyzed	3.5
Remazol Gold Yellow 3RA	
Parent	4.6
Vinyl sulfone	8.5
Hydrolyzed	7.2

Table 4: Average LC retention times of various forms of dye on the Alltech C18 analytical and C18 guard column with C18 ion-pair parameters

Analyte	Average retention time (min.)
Remazol Black B	
Parent	24.3
Vinyl sulfone	28.7
Hydrolyzed	23.5
Remazol Red RB	
Parent	28.6
Vinyl sulfone	32.5
Hydrolyzed	25.8
Remazol Gold Yellow 3RA	
Parent	27.6
Vinyl sulfone	30.1
Hydrolyzed	26.1

Zorbax Rx-C18

The Zorbax Rx-C18 analytical column was used with the same C18 reverse phase parameters as the Alltech C18, and the chromatography of the Zorbax was similar to that of the Alltech. Table 5 lists the retention times of the three major forms of the three dyes of interest. The retention times on the Zorbax Rx-C18 were, in general, shorter than those on the C18 due to the base-deactivated nature of the Zorbax Rx-C18 which may limit the retention of compounds with basic amino groups. The base-deactivation prevents irreversible adsorption of such basic compounds onto the stationary phase. However, since no such adsorption was observed with the Alltech C18 column, both columns were equally preferable for monitoring sulfonated azo dyes.

Dionex PAX-500

The PAX-500 analytical and guard columns, manufactured by Dionex Corporation (Sunnyvale, CA), did not provide an acceptable method for monitoring sulfonated azo dyes. Various attempts at gradient elution of hydrolyzed Remazol Black B with sodium hydroxide/sodium chloride solutions failed to elute the dye from the column. Attempts were then made to monitor for hydrolyzed Remazol Black B using just the PAX-500 guard column, which also resulted in no elution. Evidently

Table 5: Average LC retention times of various forms of dye on the Zorbax Rx-C18 analytical and C18 guard column with C18 reverse phase parameters

Analyte	Average retention time (min.)
Remazol Black B	
Parent	2.3
Vinyl sulfone	9.7
Hydrolyzed	7.4
Remazol Red RB	
Parent	7.1
Vinyl sulfone	9.0
Hydrolyzed	4.1
Remazol Gold Yellow 3RA	
Parent	6.1
Vinyl sulfone	9.2
Hydrolyzed	7.7

the sulfonated dyes irreversibly adsorbed to the stationary phase, or eluted so slowly as to be impractical for a monitoring method.

Quantitation of Dyes by LC

External standard curves were established for both hydrolyzed Remazol Black B and hydrolyzed Remazol Red RB to quantitate these dyes in aqueous solution. External standard solutions were prepared using LC purified, freeze-dried solid dye. Figures 16 and 17 show peak area absorbance plotted against concentration for the hydrolyzed black and red dyes, respectively. For Remazol Red RB, the peak areas plotted were the sums of the peak areas of the two major isomers of this dye. As the plots show, both curves exhibited good linearity over the entire concentration range of interest. The slopes of the lines, or mass absorbtivity, are given in units of $(\text{area} * \text{L}) / (\text{mg} * \text{cm})$, and had values of 20.8 and 56.2 for hydrolyzed Remazol Black B at 254 nm and 590 nm, respectively; and values of 31.7 and 28.3 for hydrolyzed Remazol Red RB at 254 and 525 nm, respectively.

For this external standard method to be useful, the analyst must be assured that the chromatography and detector response are reproducible over time. Repeated monitoring of a 25 μL injection of a 100 mg/L solution of 4-amino-3-hydroxy-

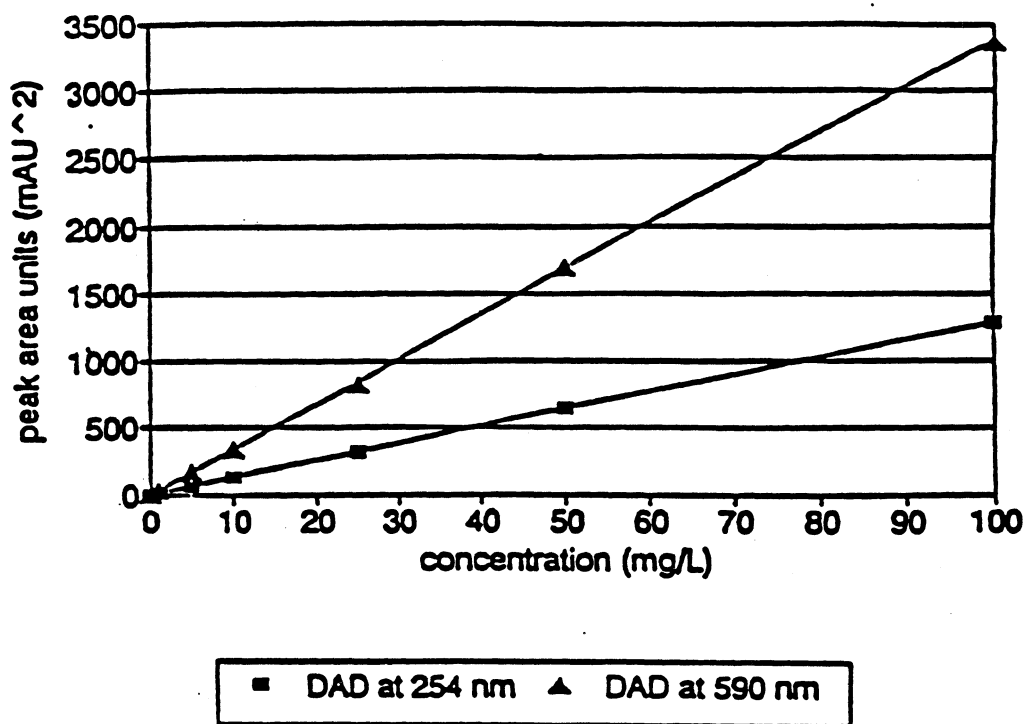


Figure 16: External standard curve for purified hydrolyzed Remazol Black B measured by liquid chromatography with diode array detection (LC/DAD) (mAU² refers to milli-absorbance units squared).

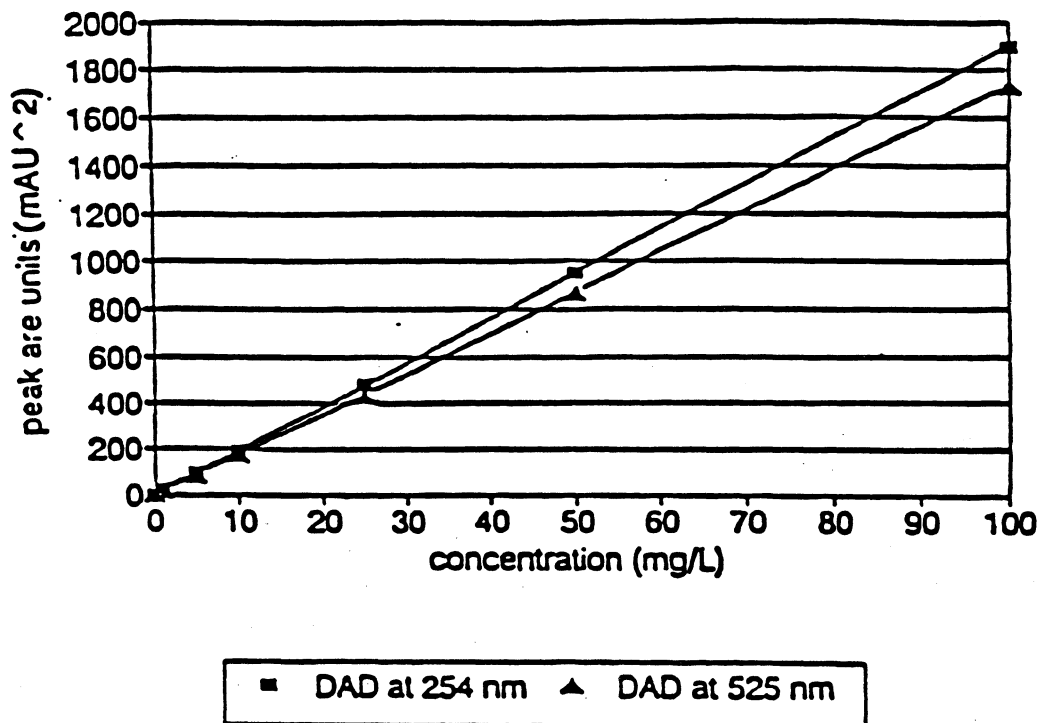


Figure 17: External standard curve for purified hydrolyzed Remazol Red RB, including all major isomers, measured by liquid chromatography with diode array detection (LC/DAD) (mAU² refers to milli-absorbance units squared).

1-naphthalene sulfonic acid showed a fairly constant detector response over time at 254 nm and 450 nm (Figure 18). The average standard deviation in peak area for this compound, obtained by adding the variances and taking the square root, was 5.2 peak area units at 254 nm and 7.4 peak area units at 450 nm.

Instrumental detection limits for the dyes were taken as the mean concentration of dye corresponding to a peak twice the height of the noise, or variation in the baseline. The detection limit of a dye obtained by this method will vary depending on the detection wavelength of the diode array detector. Analyses of mixtures of compounds were generally performed at 254 nm, but the detection limit of a given dye can be minimized by performing the analysis at the wavelength at which the dye exhibits maximum absorbance, which will also reduce noise due to contaminants which absorb at lower wavelengths. Mean variation in baseline, reported in units of milli-Absorbance (mAU), was 4.7 mAU at 254 nm, 3.6 mAU at 525 nm, and 4.0 mAU at 590 nm. Detection limits of dye corresponding to twice these values are reported in Table 6 at 254 nm and 597 nm for hydrolyzed Remazol Black B, and 254 nm and 525 nm for hydrolyzed Remazol Red RB.

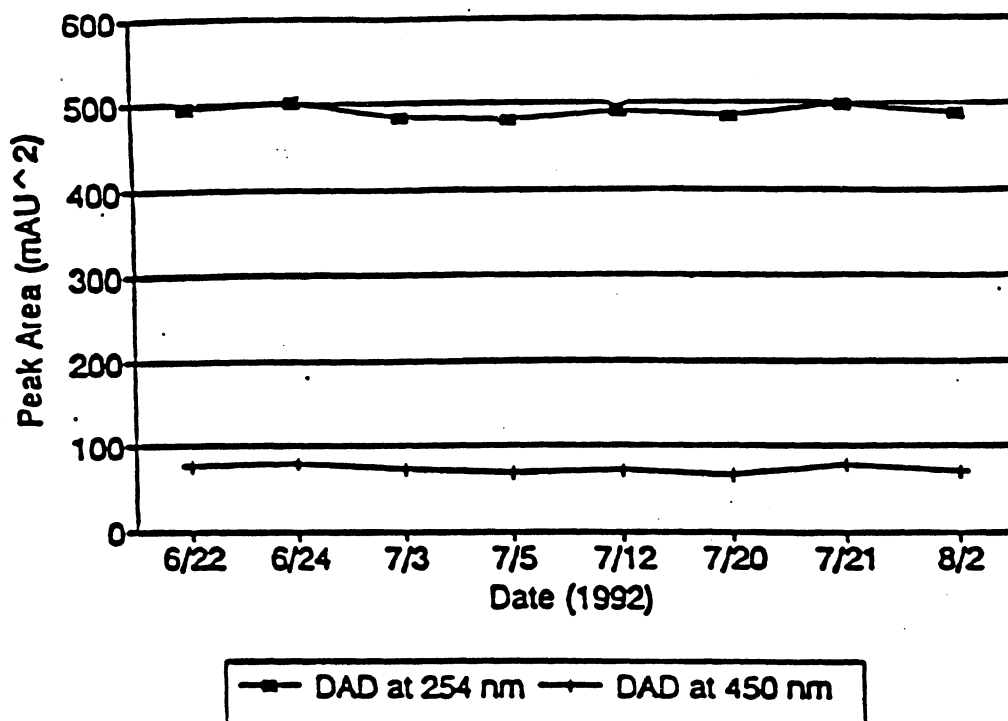


Figure 18: Quality control data for the response of the diode array detector over time: 25 μ L injection of 100 mg/L 4-amino-3-hydroxy-1-naphthalene sulfonic acid at 254 nm and 450 nm were monitored.

Table 6: Instrumental detection limits (mg/L)for hydrolyzed Remazol Black B and hydrolyzed Remazol Red RB using LC/DAD for three detection wavelengths.

Dye	Detection Limit (mg/L)		
	254 nm	525 nm	590 nm
Hydrolyzed Remazol Black B	8.0	--	2.6
Hydrolyzed Remazol Red RB	5.3	4.9	--

LC/MS of Remazol Black B and Remazol Red RB

Electrospray LC/MS analyses, in both negative-ion and positive-ion, were performed in order to confirm/elucidate the structure of Remazol Black B and Remazol Red RB. This was performed off-line so that the resulting mass spectra are composites of all compounds in the sample. Flow fast atom bombardment mass spectrometry (FAB-MS) was also performed on Remazol Red RB. The size, polarity, and high salt concentrations of sulfonated azo dyes made them a special challenge for obtaining good mass spectra. Fragmentation of the molecule, possibly at azo or carbon-nitrogen bonds, and the formation of adduct ions with salts in the sample made the recognition of molecular ions difficult.

Positive-ion electrospray LC/MS gave strong signals of hydrolyzed Remazol Black B (Figure 19) and was able to confirm the structure of the hydrolyzed dye. The molecular ion occurred as an adduct with three sodium ions at m/z 810. Positive-ion electrospray mass spectrometry of hydrolyzed Remazol Red RB gave little detector response, but negative-ion ES gave strong signals at m/z 687 and 324 (Figure 20). As the voltage of the electrospray interface increased, the relative proportions of these two m/z values did not markedly

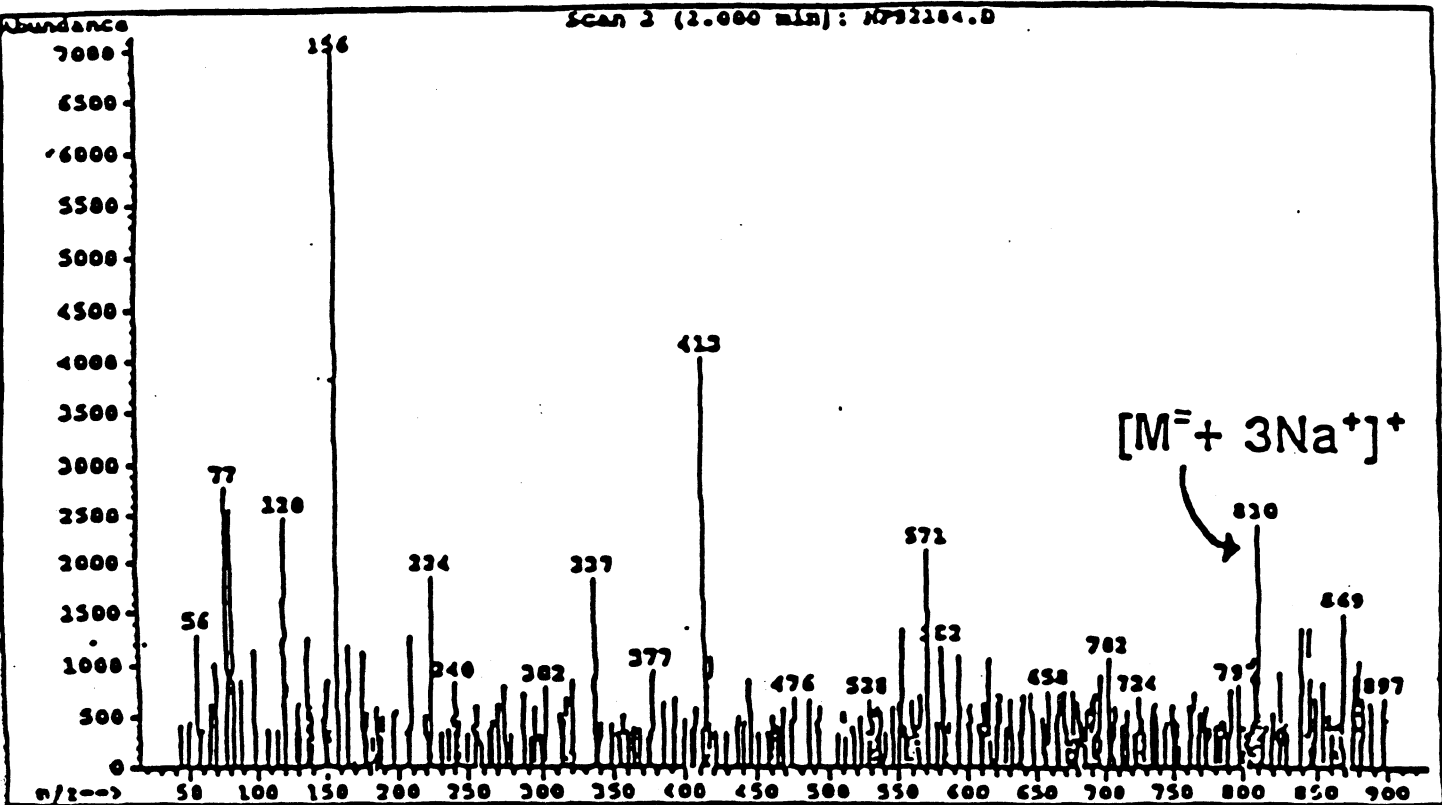


Figure 19: Positive-ion electrospray mass spectrum of hydrolyzed Remazol Black B (MW=741 amu). Major sodium adduct was m/z 810, corresponding to $[M^- + 3Na^+]^+$.

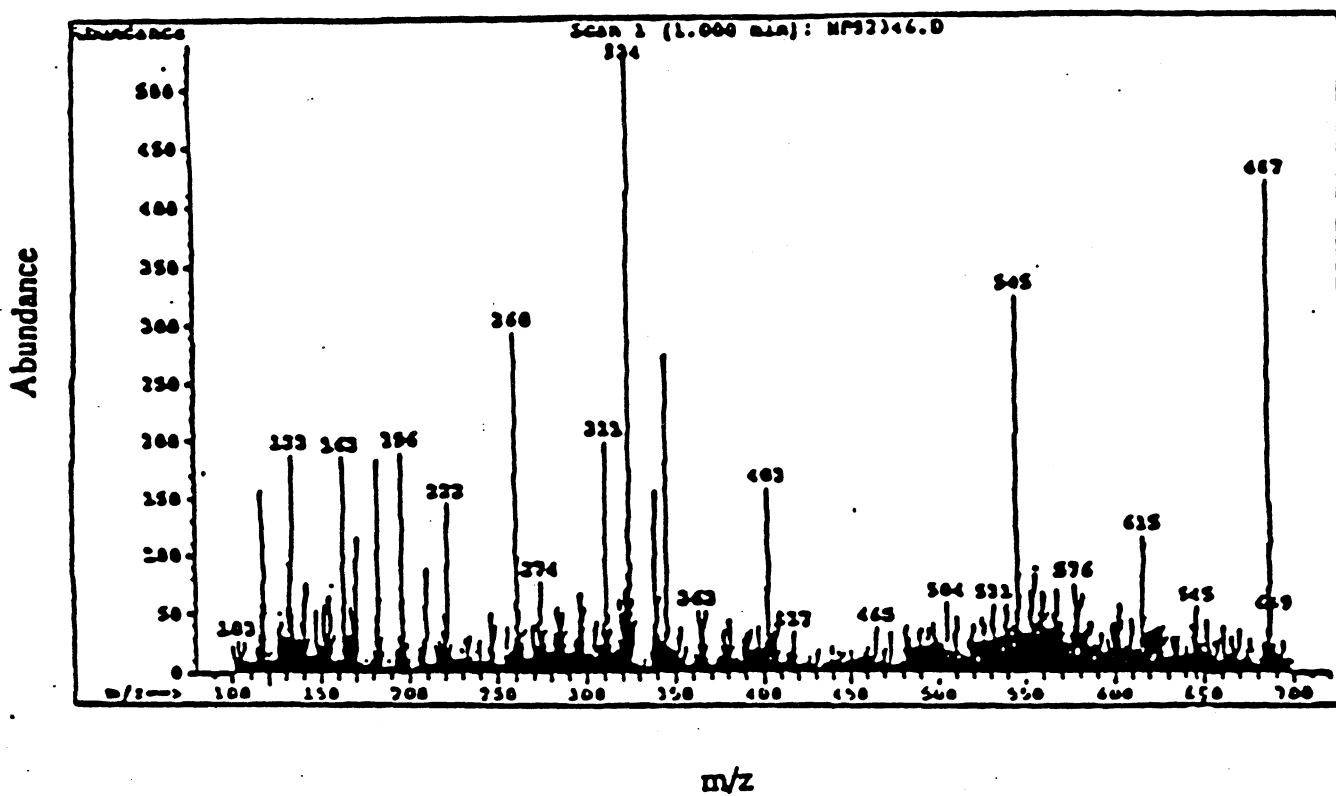


Figure 20: Negative-ion electrospray mass spectrum of hydrolyzed Remazol Red RB.

change, suggesting that 324 is not a daughter product of 687. Attempts were made to explain these m/z values based on knowledge that the dye had a vinyl sulfone reactive group coupled by azo linkage to H-acid, and a triazine reactive group bonded to the 1-amino group of H-acid. Thus far, however, no structure has been proposed that matches the mass spectral data.

Monitoring for Dye Reduction Products

Aqueous solutions of Remazol Black B, Remazol Red RB, or Remazol Gold Yellow 3RA, prepared with solid dye from the manufacturer, then chemically reduced by the addition of sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$). The resulting solutions contained excess reducing agent, and were yellow in color. These solutions were analyzed by LC/DAD, GC/MS, and LC/MS to detect and determine the structures of reduction products. LC/DAD analysis of reduced dyes was performed on the Alltech C18 and Zorbax Rx-C18 analytical columns with reverse parameters, and on the Alltech C18 analytical column with ion-pair parameters.

Reduction of Remazol Black B

Liquid chromatography of reduced Remazol Black B showed two major reduction products (Figure 21) called B-1 and B-X-

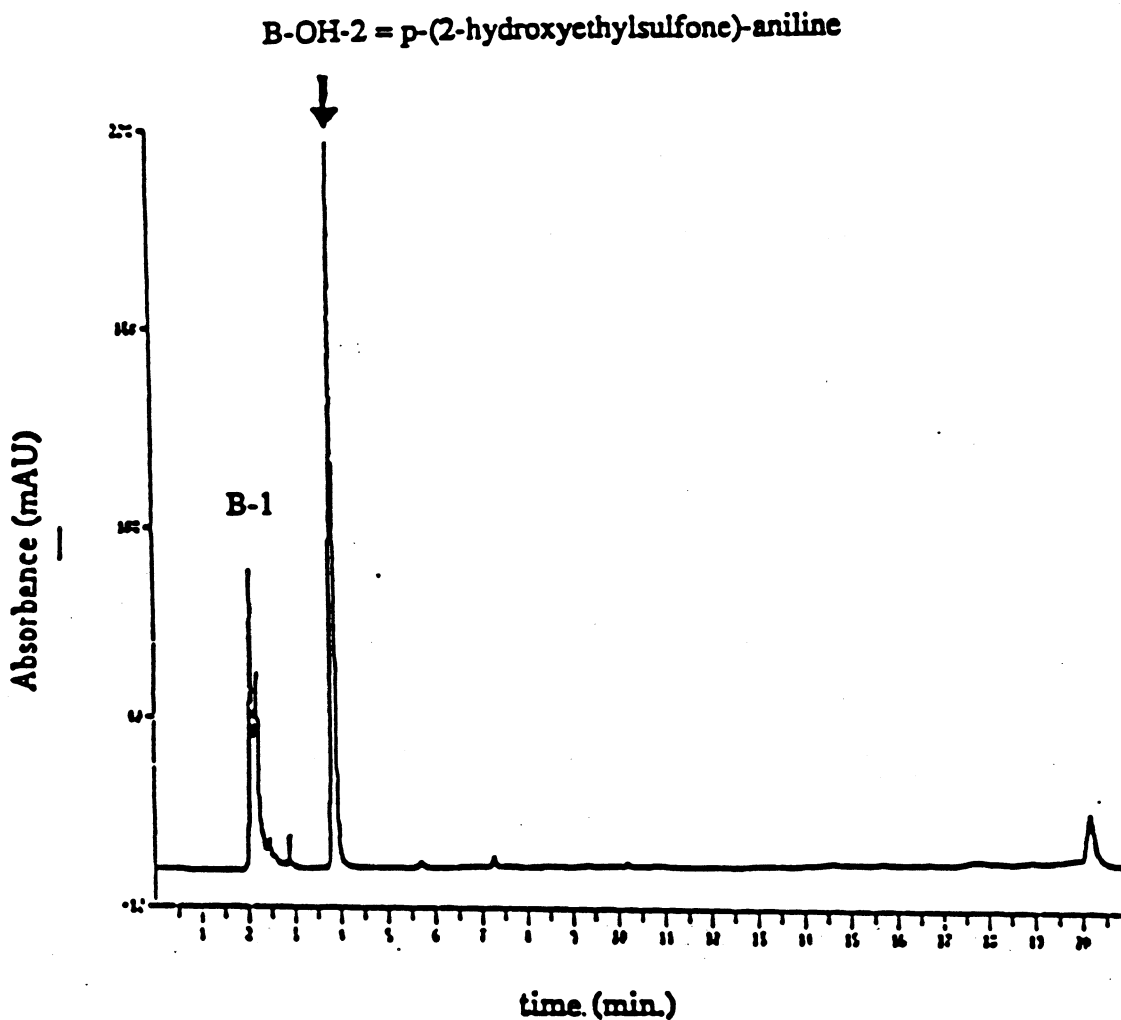


Figure 21: LC chromatogram of reduced hydrolyzed Remazol Black B, showing peaks of the two major reduction products. B-1 is probably a derivative of the central naphthalene-based chromophore of the dye, while B-OH-2 is p-(2-hydroxyethylsulfone)-aniline.

2, where X is Par, VS, or OH depending on whether the dye is in the parent, vinyl sulfone, or hydrolyzed form, respectively. The retention times of these compounds are listed in Table 7, and the UV/Vis spectra are shown in Figure 22. B-1, which was common to all forms of Remazol Black B, was suspected to be a derivative of the sulfonated central naphthalene-based chromophore of the dye. Another azo dye, Acid Black 1, contained the same naphthalene-based chromophore and also yielded B-1 upon reduction by hydrosulfite, as evidenced by identical retention time and UV/Vis spectra (Figure 23). This is strong evidence that B-1 is in fact a derivative of the sulfonated naphthalene-based chromophore. Using the C18 reverse phase parameters this product eluted close to the dead volume time of the column, indicating a very polar product, but C18 ion-pair parameters increased the retention time greatly.

B-X-2 was identified as being the substituted aniline derived from the fiber reactive groups of the molecule. Specifically, GC/MS of reduced hydrolyzed Remazol Black B extracted into methylene chloride confirmed the structure of p-(2-hydroxyethylsulfone)-aniline (Figure 24) as a reduction product of the dye; this product was present in both the acid and base/neutrals fraction of the extract. Two

Table 7: Typical LC retention times of reduction products of Remazol Black B, Remazol Red RB, and Remazol Gold Yellow 3RA.

Reduction Product	Alltech C18 Reverse Phase Parameters	Alltech C18 Ion-Pair Parameters	Zorbax Rx-C18 Reverse Phase Parameters
B-1	2.3	13.2	2.0
R-1	2.5	14.2	2.1
Y-1	2.7	11.7	3.5
B-Par-2			
R-Par-2	2.6	5.5	2.7
Y-Par-2			
B-VS-2			
R-VS-2	5.0	4.9	6.6
Y-VS-2			
B-OH-2			
R-OH-2	3.3	3.3	3.1
Y-OH-2			

(B,R,Y)-Par-2 = p-(2-Sulfatoethylsulfone)-aniline

(B,R,Y)-VS-2 = p-(2-Vinylsulfone)-aniline

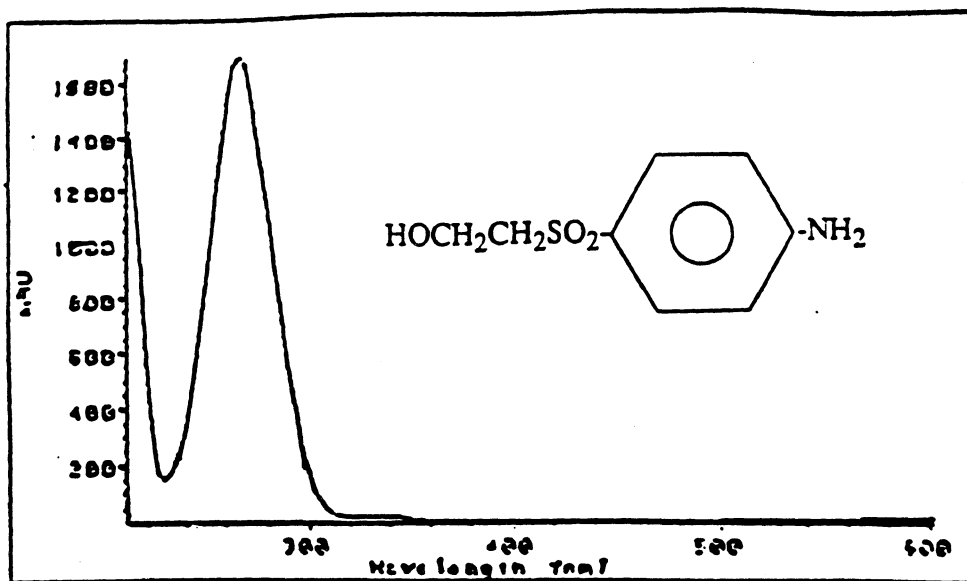
(B,R,Y)-OH-2 = p-(2-Hydroxyethylsulfone)-aniline

B- refers to products derived from the reduction of Remazol Black B

R- refers to products derived from the reduction of Remazol Red RB

Y- refers to products derived from the reduction of Remazol Gold Yellow 3RA

(a)



(b)

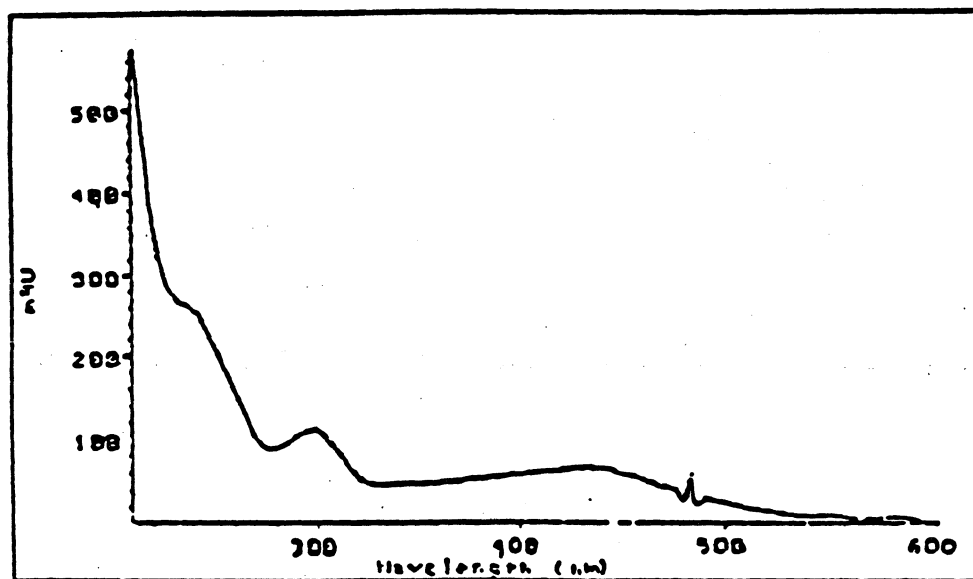


Figure 22: UV/Visible absorbance spectra of reduction products of hydrolyzed Remazol Black B: (a) B-OH-2 which is p-(2-hydroxyethylsulfone)-aniline, and (b) product called B-1, which is probably a derivative of the central naphthalene-based chromophore of the dye.

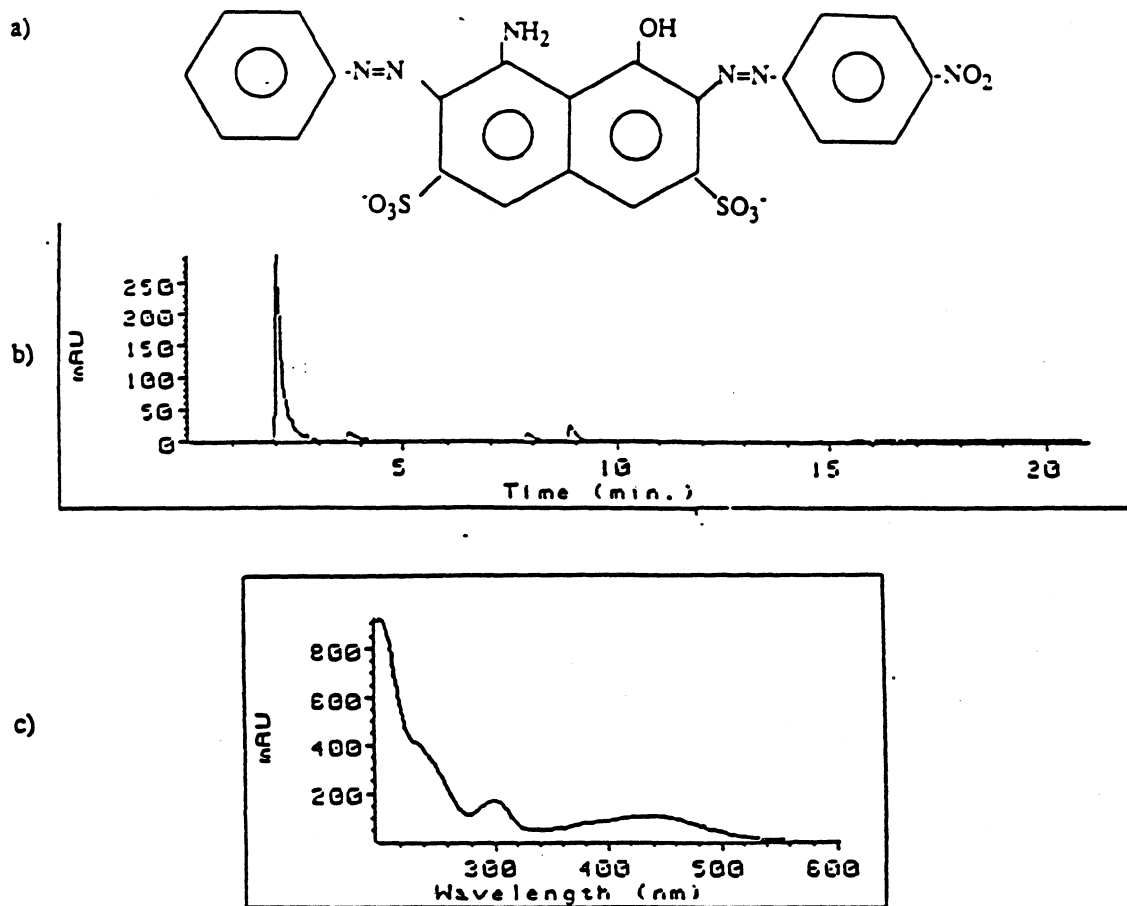


Figure 23: (a) Structure of Acid Black 1. (b) Chromatogram of reduced Acid Black 1 under Alltech C18 reverse phase parameters, showing peak at 2.3 minutes. (c) UV/Visible absorbance spectrum of compound which elutes at 2.3 minutes. This compound shares the retention time and UV/Visible spectrum of B-1, the reduction product of Remazol Black B, suggesting that B-1 is a derivative of the central naphthalene-based chromophore which the two dyes have in common.

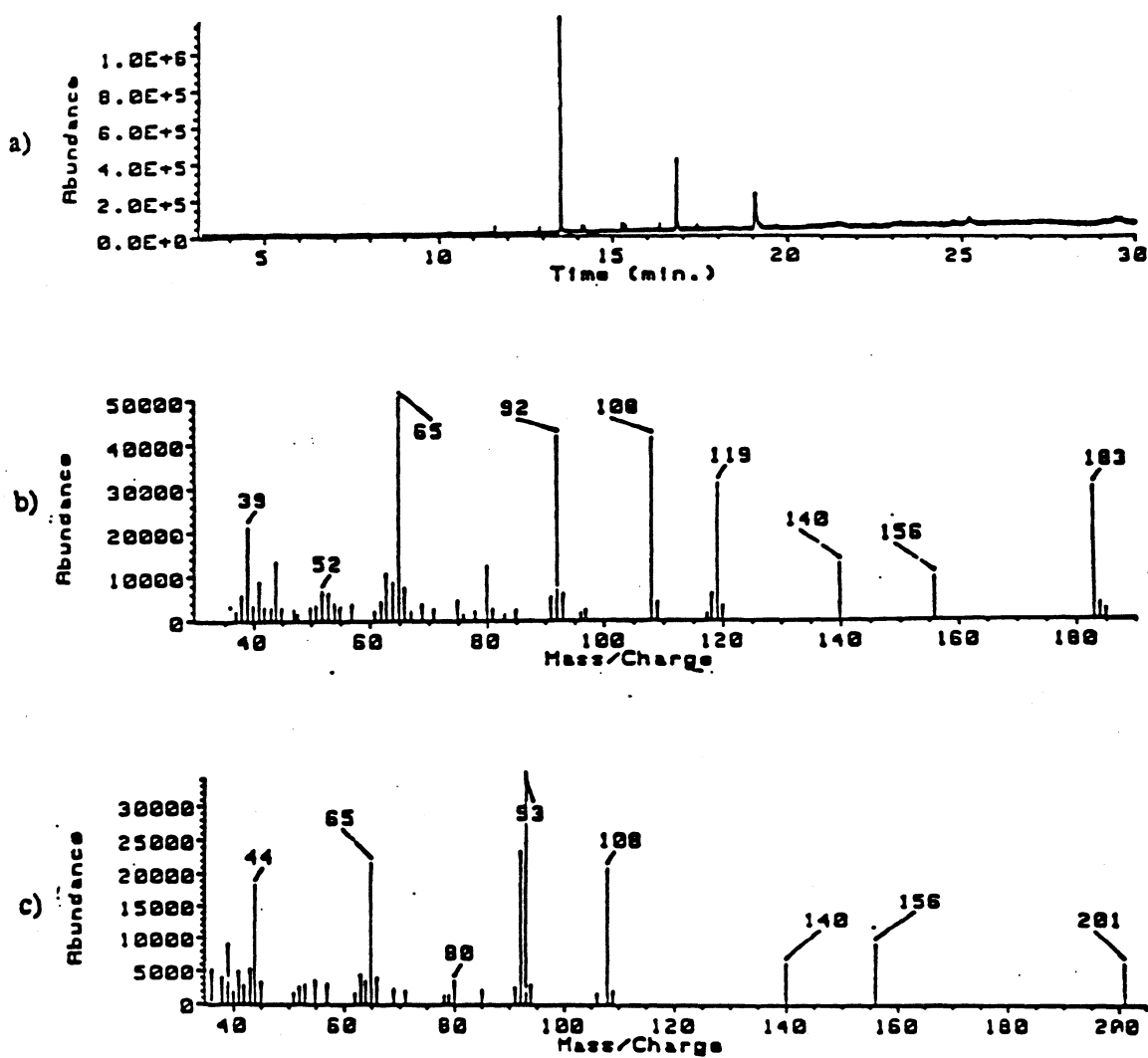


Figure 24: (a) GC chromatogram of base/neutral fraction of methylene chloride extract of reduced hydrolyzed Remazol Black B. (b) Mass spectrum of compound which elutes at 16.8 minutes, p-(2-hydroxyethylsulfone)-aniline minue H_2O . (c) Mass spectrum of compound which elutes at 19 minutes, p-(2-hydroxyethylsulfone)-aniline.

chromatographic peaks indicate presence of this compound: the molecular ion at m/z 201, and another peak at m/z 183 which probably represents the compound after loss of H_2O in the hot GC injector.

Positive-ion electrospray LC/MS of reduced hydrolyzed Remazol Black B revealed a major peak at m/z 223 (Figure 25).

If *p*-(2-hydroxyethylsulfone)-aniline were to form an adduct with potassium after loss of water, or with sodium, the resulting m/z values would be 222 and 224, respectively. Thus the observed peak at 223 could represent a variation of one of these adducts.

Oxidation of Reduced Remazol Black B

When reduced with excess sodium hydrosulfite, aqueous solutions of Remazol Black B took on a residual yellow color, possibly due to the presence of aromatic amines. However, upon the addition of a few milliliters of 30% hydrogen peroxide (H_2O_2), a strong oxidizing agent, the color of the solutions turned bluish-purple. While this experiment was strictly qualitative, the observed phenomenon may indicate that one or more reduction products can re-oxidize into larger conjugated systems; possibly by the oxidation of amino groups into nitro or nitroso groups. It is not expected that the azo bond could reform.

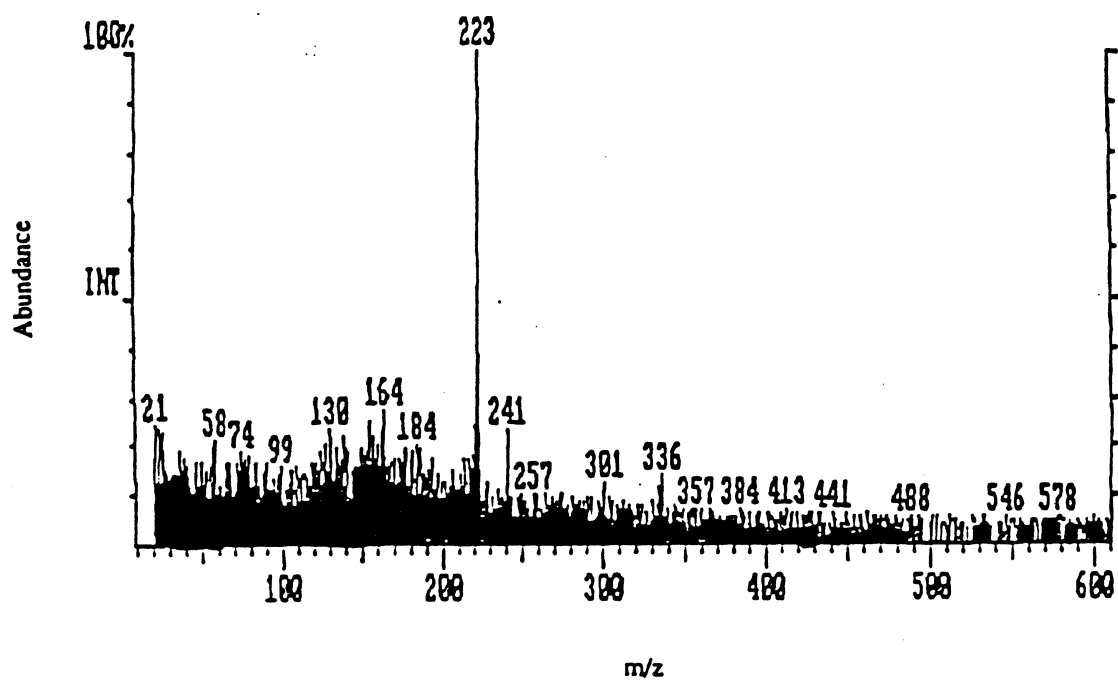


Figure 25: Positive-ion electrospray mass spectrum of reduced hydrolyzed Remazol Black B, showing major peak at m/z 223.

Reduction of Remazol Red RB

The reduction of Remazol Red RB by excess hydrosulfite yielded two major reduction products as evidenced by LC (Figure 26), called R-1 and R-X-2. Retention times of these peaks on the different columns and LC parameters are listed in Table 7, and UV/Vis spectra are shown in Figure 27. R-OH-2 has been confirmed by several methods to be the same product, p-(2-hydroxyethylsulfone)-aniline, that was also a reduction product of hydrolyzed Remazol Black B. The UV/Vis spectra and LC retention times of R-OH-1 and B-OH-1 are identical, and both GC/MS and LC/MS of reduced hydrolyzed Remazol Red RB indicated the structure of p-(2-hydroxyethylsulfone)-aniline (Figure 28). R-1 is suspected to be a derivative of the sulfonated naphthalene-triazine part of the molecule. Once again, ion-pair chromatography was necessary to obtain satisfactory retention times of this extremely polar product.

Reduction of Remazol Gold Yellow 3RA

The reduction of Remazol Gold Yellow 3RA yielded 2 major reduction products as evidenced by LC (Figure 29), called Y-1 and Y-X-2. The retention times of these peaks are listed in Table 7, and the UV/Vis spectra are shown in Figure 30. The UV/Vis spectra and LC retention times of Y-X-2 indicate

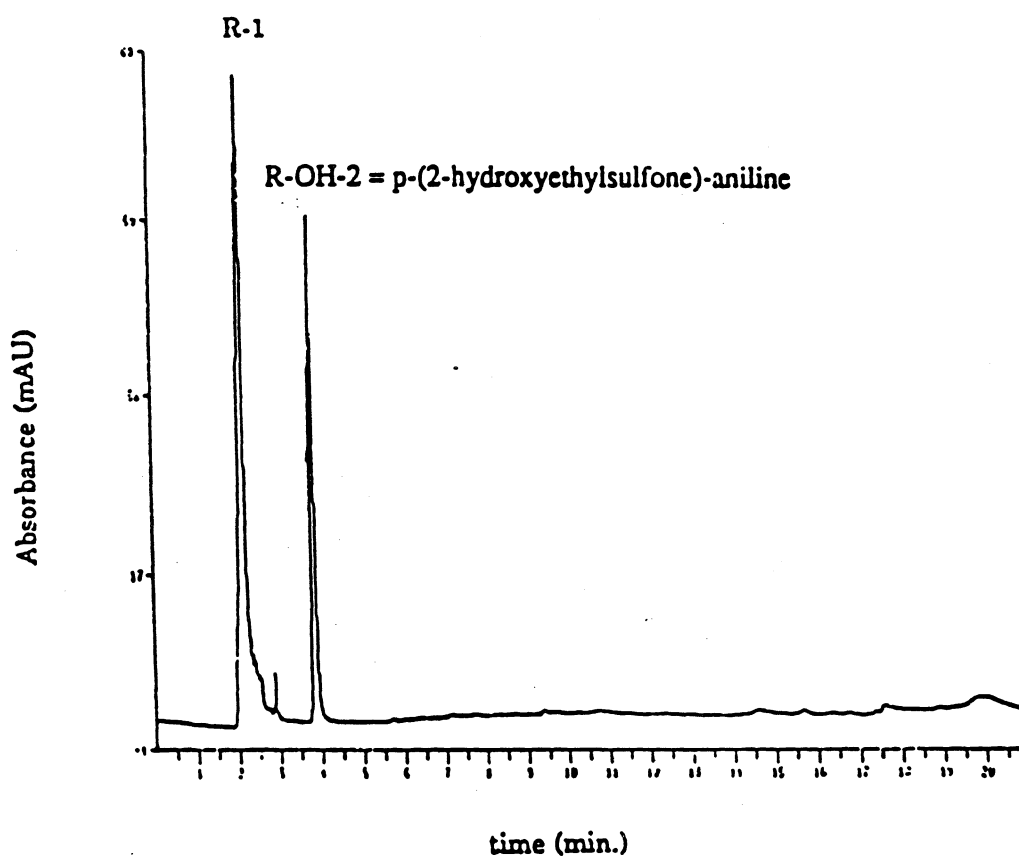


Figure 26: LC chromatogram of reduced hydrolyzed Remazol Red RB performed with Alltech C18 parameters using detection wavelength of 254 nm, showing the two major reduction products. R-1 is probably a derivative of the naphthalene-triazine-based chromophore, while R-OH-2 is p-(2-hydroxyethylsulfone)-aniline.

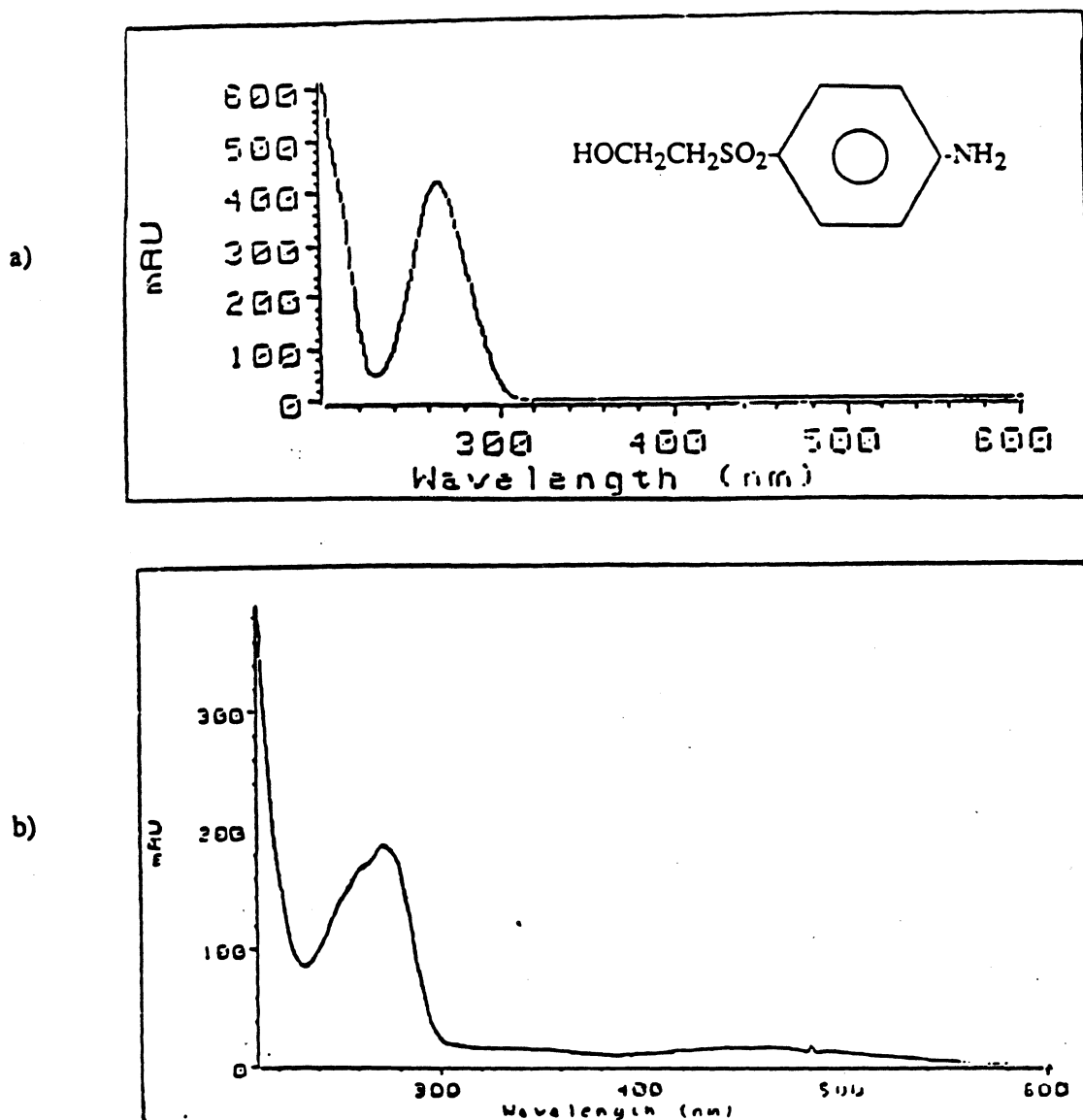


Figure 27: UV/Visible absorbance spectra of reduction products of hydrolyzed Remazol Red RB: (a) R-OH-2 which is p-(2-hydroxyethylsulfone)-aniline, and (b) product called R-1, probably a derivative of the naphthalene-triazine-based portion of the dye.

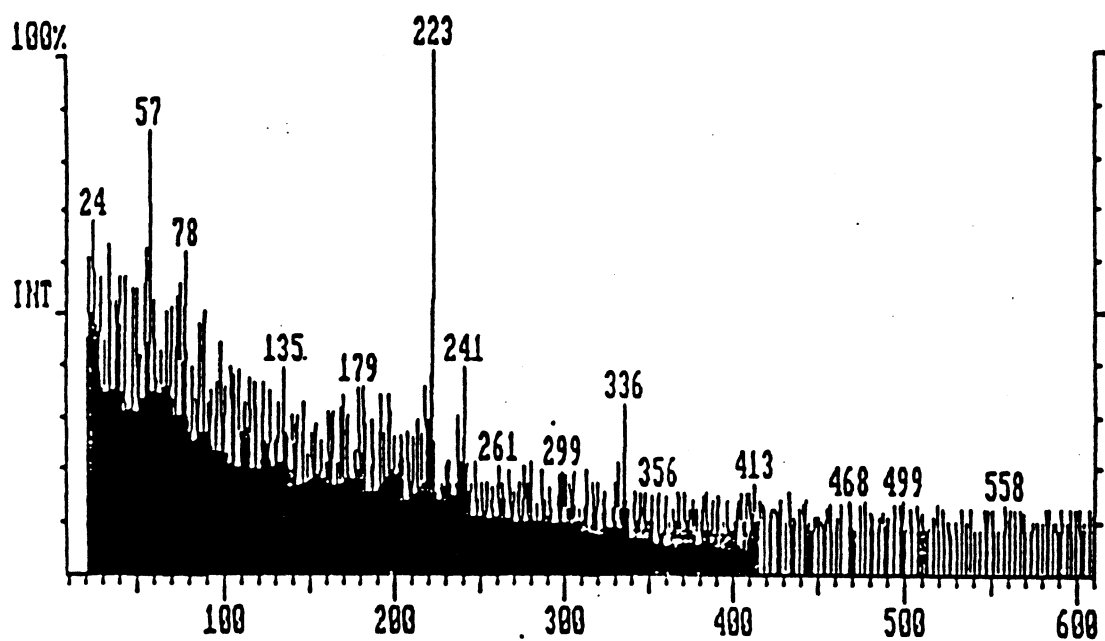


Figure 28: Positive-ion electrospray mass spectrum of reduced hydrolyzed Remazol Red RB, showing major peak at m/z 223.

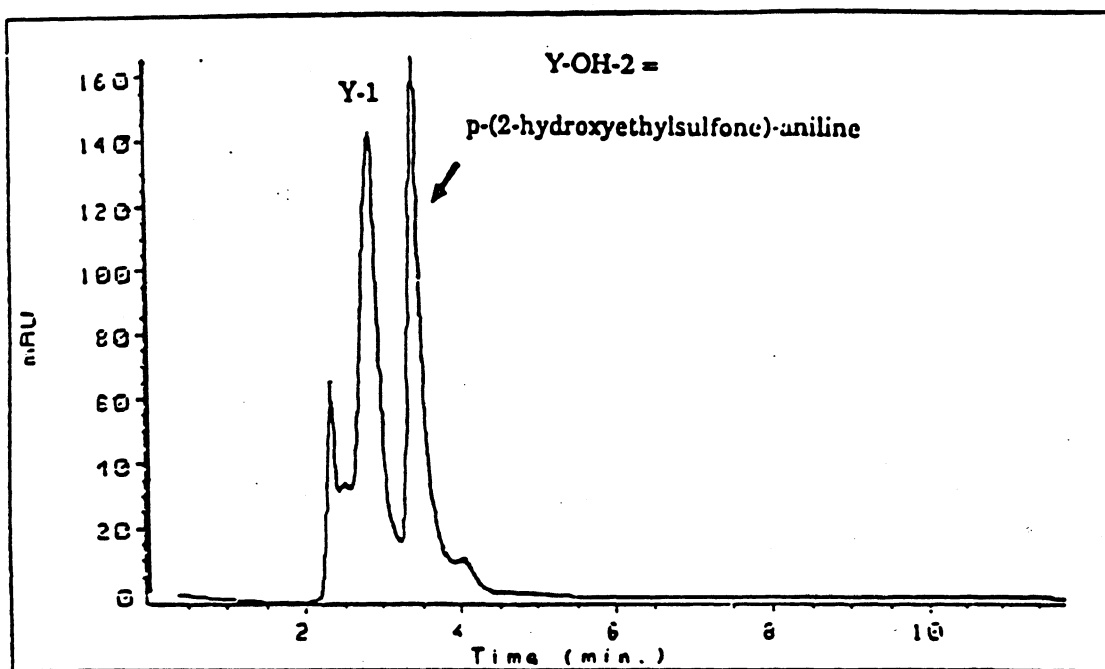
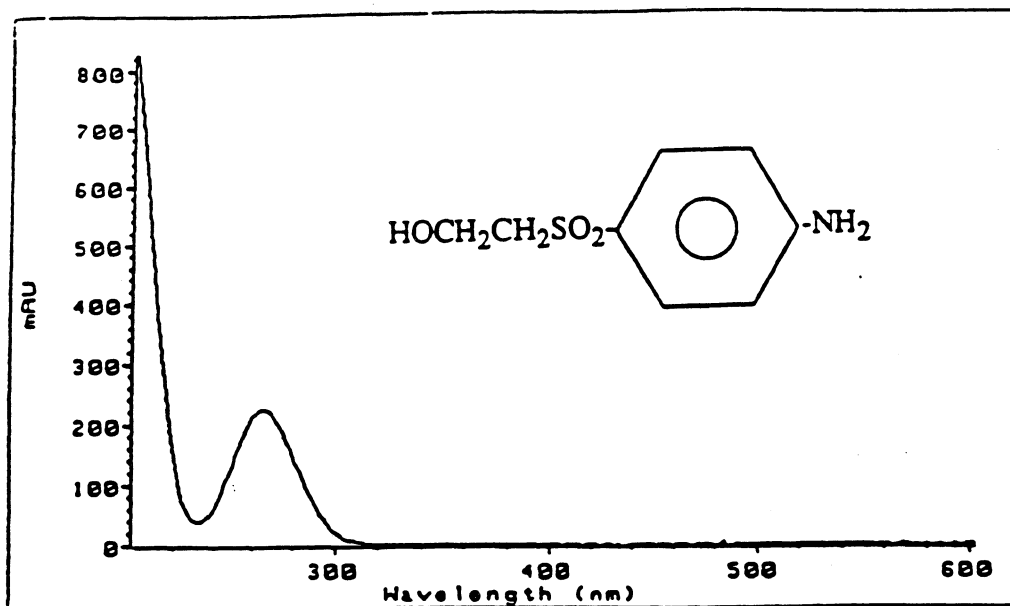


Figure 29: LC chromatogram of reduced hydrolyzed Remazol Gold Yellow 3RA performed on Alltech C18 parameters using detection wavelength of 254 nm, showing the two major reduction products. Y-1 is probably a derivative of the central naphthalene-based chromophore, while Y-OH-2 is p-(2-hydroxyethylsulfone)-aniline (mAU refers to mill-absorbance units).

a)



b)

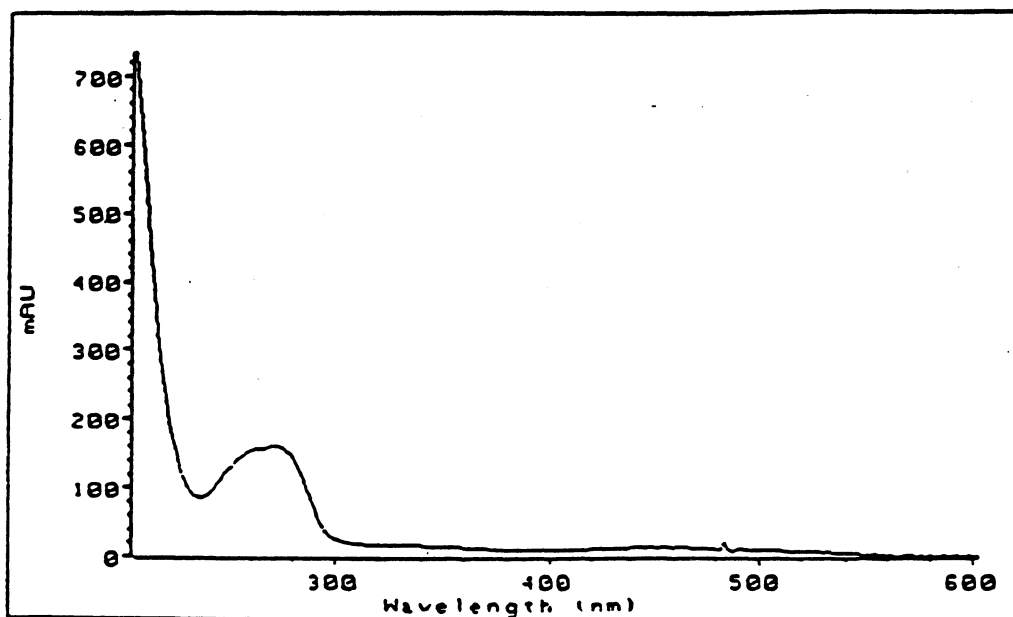


Figure 30: UV/Visible absorbance spectra of reduction products of hydrolyzed Remazol Gold Yellow 3RA: (a) Y-OH-2 which is p-(2-hydroxyethylsulfone)-aniline, and (b) product called Y-1 which is probably a derivative of the naphthalene-based part of the dye (mAU refers to mill-absorbance units).

that it is the same reduction product as B-X-2 and R-X-2, p-(2-hydroxyethylsulfone)-aniline. Y-1 is suspected to be a derivative of the sulfonated naphthalene-based chromophore of the dye, which is best separated on the C18 ion-pair parameters. It should be noted that Y-1 does not share the retention time or UV-Vis spectra of B-1.

Sulfonated reduction products, like sulfonated dyes, present a special mass spectrometric challenge due to their size and polarity. These compounds are probably amines and were likely responsible for the yellow color of reduced dye solutions. At this time, no satisfactory GC/mass spectra or LC/mass spectra have been obtained of the sulfonated reduction products B-1, R-X-1, or Y-1. Dye chemists have indicated that the simplest naphthalene-based amines expected from the reductive cleavage of azo bonds coupled to H-acid have not, in fact, been observed as stable reduction products of these dyes (Riefe, 1992, Parry, 1992). These naphthalene-based products may undergo some type of oxidation or rearrangement reaction following reductive cleavage. It is hoped that continuing work on LC/MS or reduced dyes may elucidate the structures of these compounds. In particular, on-line LC/MS may reduce suppression of ionization caused by the high concentrations of reducing agents and other salts in the

sample. Newer mass spectrometric techniques such as flow FAB and capillary zone electrophoresis (CZE) may also produce results.

Characterization of Navy 106 Dye Washwater

The results described above have been for laboratory-prepared solutions made with powdered dye from the manufacturer. After the analytical procedures were established, these procedures were applied to actual wastewater samples, before and after anaerobic and anaerobic/aerobic biological treatment. Chemically, Navy 106 is very different from aqueous solutions prepared with dye from the manufacturer; only a few percent of the TOC was due to the dyes in Navy 106 (Powell, 1991), and Navy 106 contains significant concentrations of other compounds such as silicates and surfactants (e. g. pine oil, tributylphosphate, and isopropyl alcohol). As mentioned previously, the TOC of Navy 106 Dye Washwater ranged between 289 mg/L and 385 mg/L, with ADMI Color Value between 9461 and 10108 and a pH between 10.5 and 12.

Untreated Navy 106

All three dyes of interest were detected in fresh untreated Navy 106 Dye Washwater obtained directly from the textile dyeing facility (Figure 31). Both Remazol Black B and Remazol Gold Yellow 3RA were detected in their hydrolyzed forms, and the concentration of hydrolyzed Remazol Black B varied from 5 to 40 mg/L in undiluted washwater. It should be noted that the retention times of Remazol Red RB in Navy 106 do not correspond exactly to retention times observed with laboratory-prepared solutions. This could be because of partial hydrolysis; the Remazol reactive group may be hydrolyzed while the reactive group on the triazine may not be. Alternately, the differences in sample composition may cause different behavior of Remazol Red RB in Navy 106 than in lab-prepared solutions (e. g. salts, pH, competing organics). However, the dye can be still identified from the distinctive UV/Vis spectrum.

GC/MS of untreated Navy 106 Dye Washwater reveals the presence of a variety of compounds (Figure 32). Major components tentatively identified by library peak-matching include the following: 1-alpha-terpineol (Figure 33), endo-fenchol (Figure 34), tributylphosphoric acid (Figure 35), limonene (Figure 36) and substituted cyclohexanols,

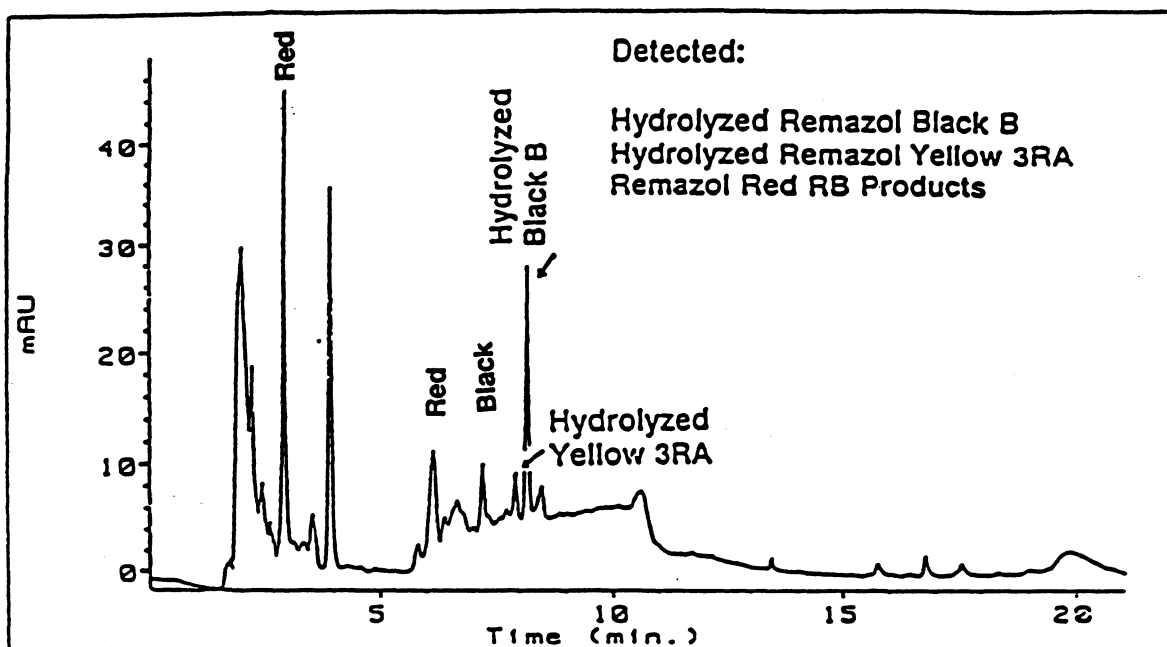


Figure 31: LC chromatogram of untreated Navy 106 washwater on Alltech C18 reverse phase parameters, using detection wavelength of 254 nm and showing all three azo dyes of interest (mAU refers to milli-Absorbance units).

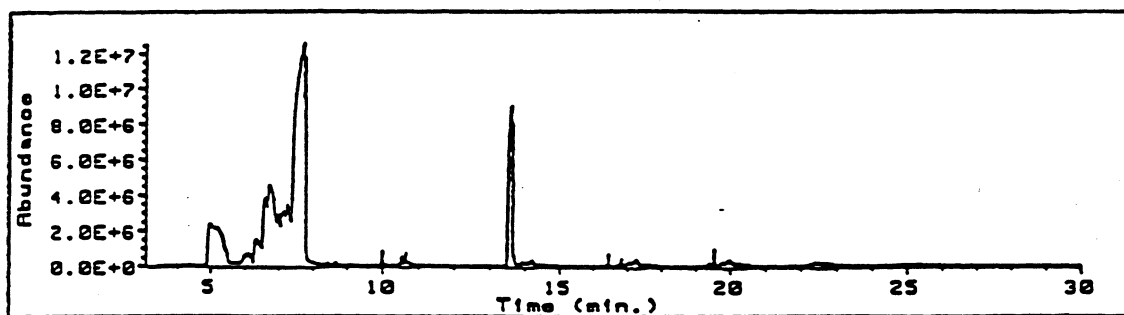


Figure 32: GC/EI-MS chromatogram of base/neutrals methylene chloride extract of untreated Navy 106 washwater, showing a variety of volatile and semi-volatile organic compounds detected.

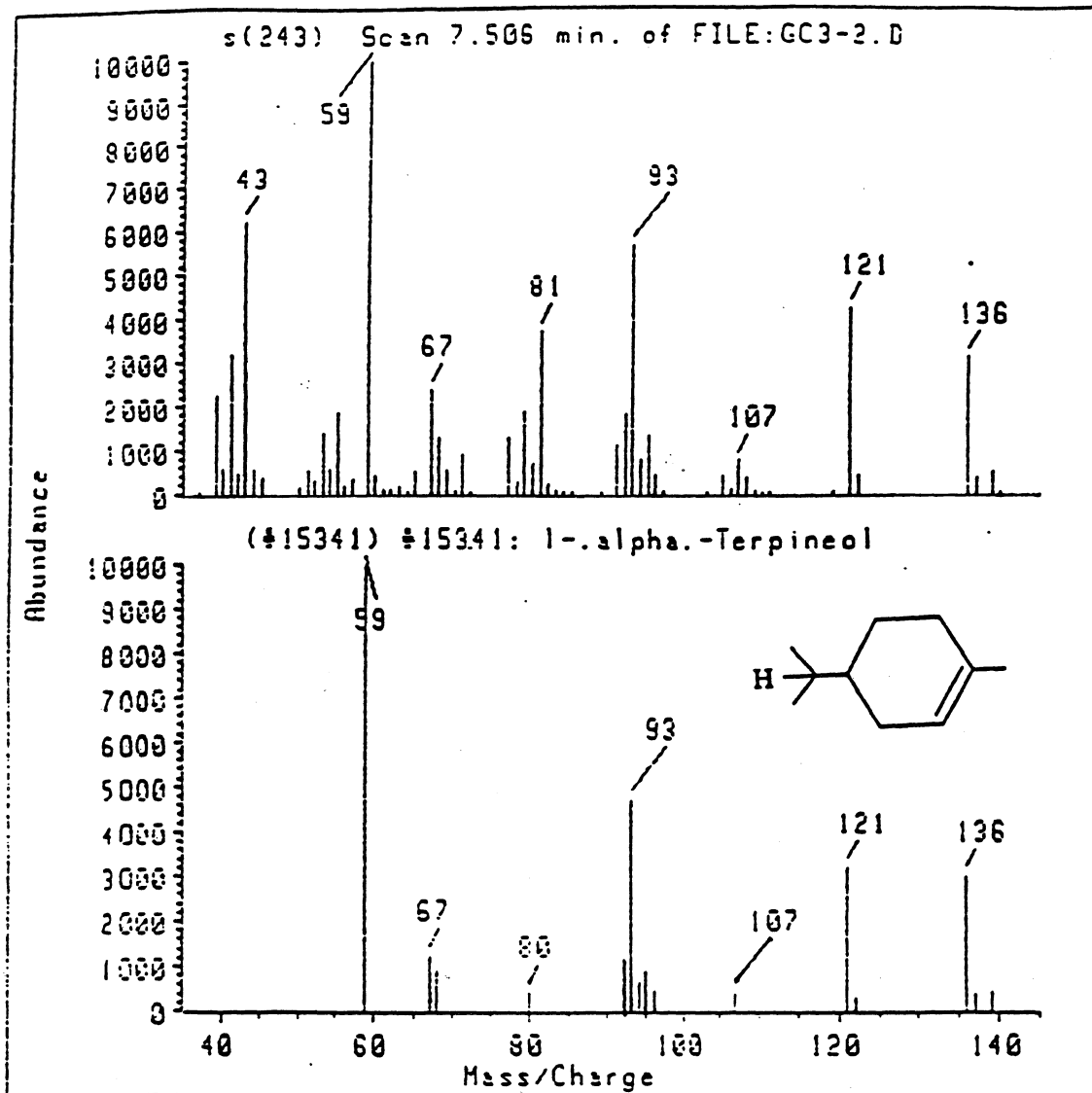


Figure 33: Library match of compound detected in base/neutral methylene chloride extract of untreated Navy 106 dye washwater to 1- α -terpineol.

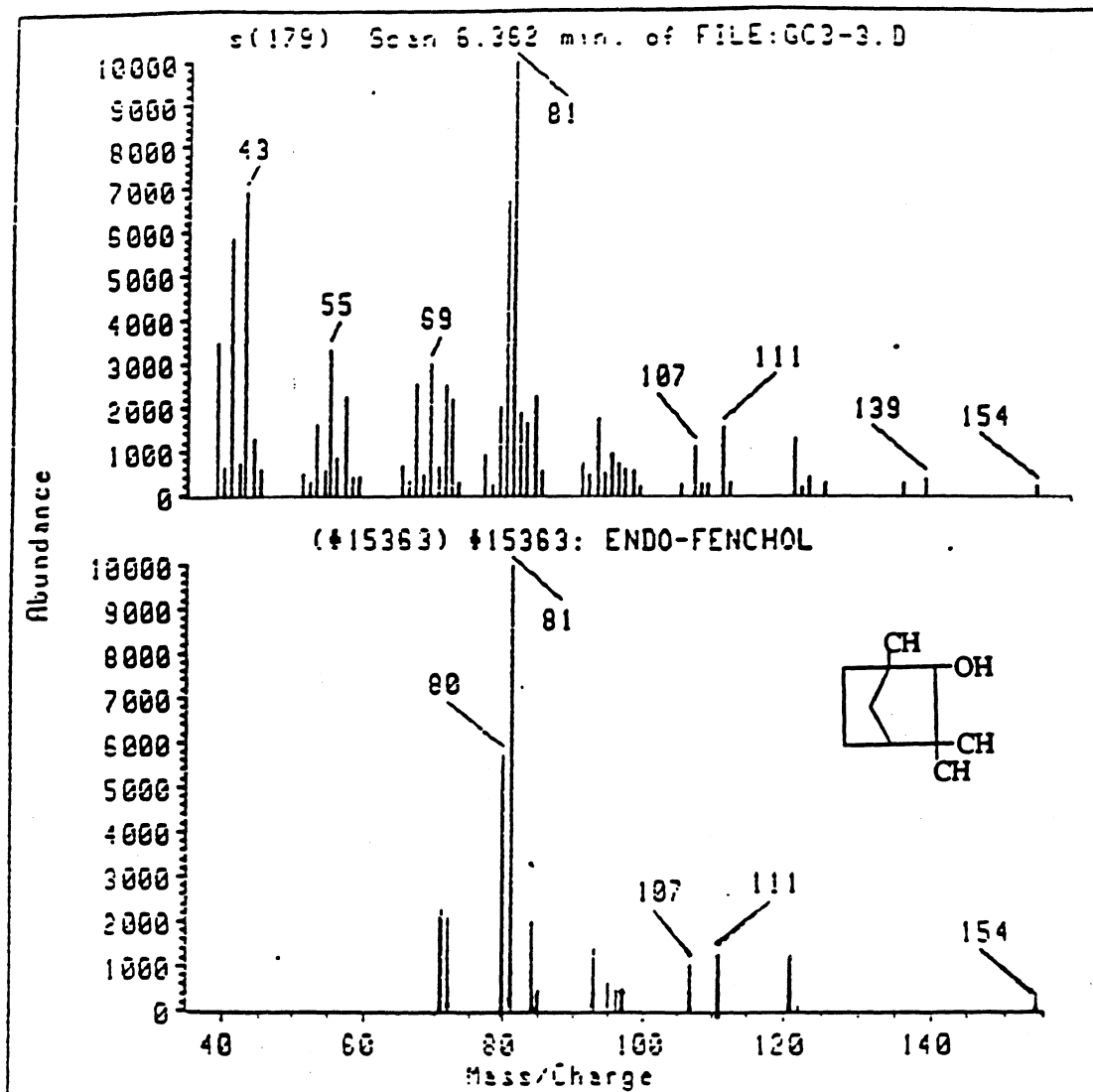


Figure 34: Library match of compound detected in base/neutral methylene chloride extract of untreated Navy 106 dye wastewater to endo-fenchol.

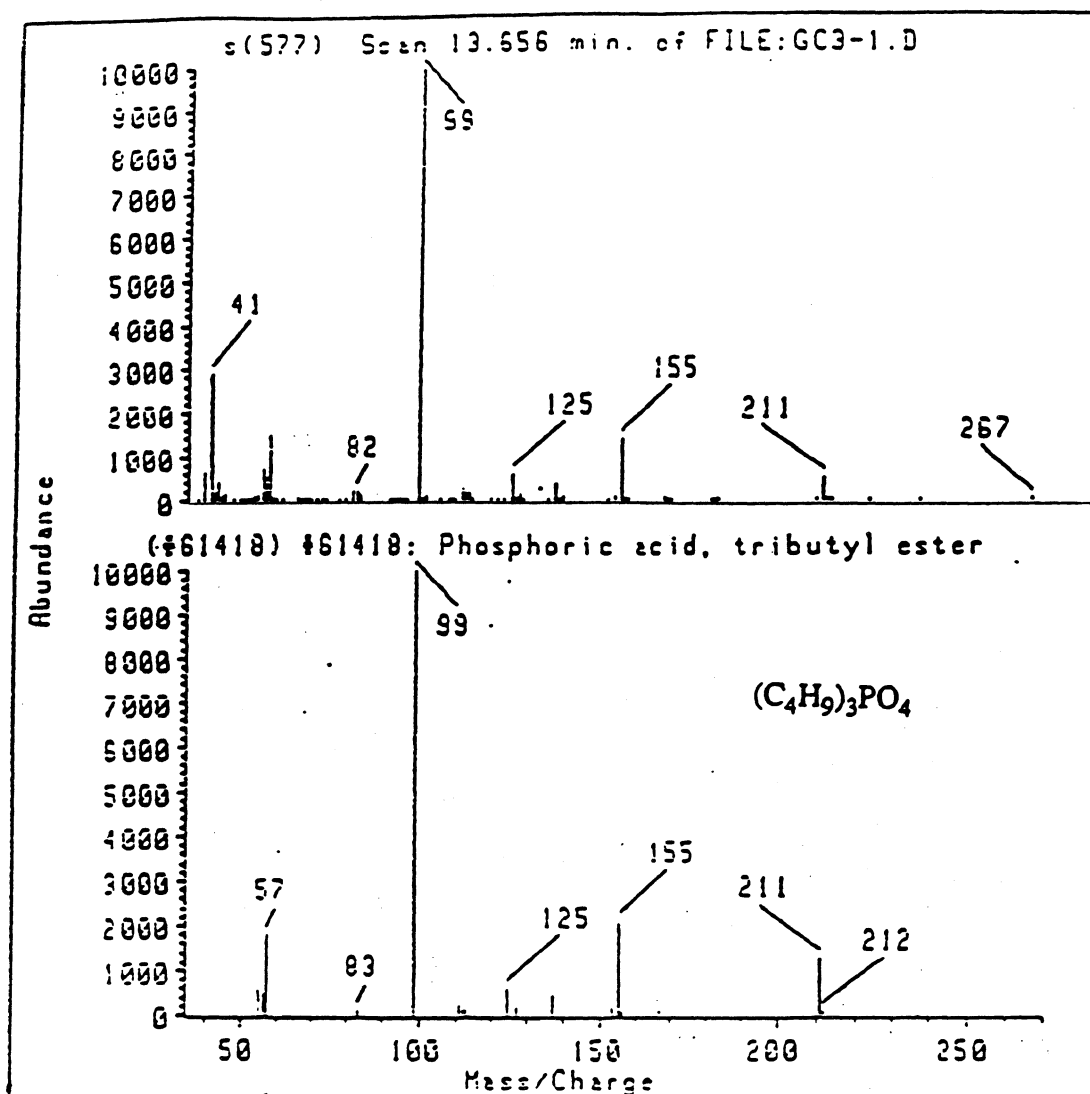


Figure 35: Library match of compound detected in base/neutral methylene chloride extract of untreated Navy 106 dye washwater to tributylphosphoric acid.

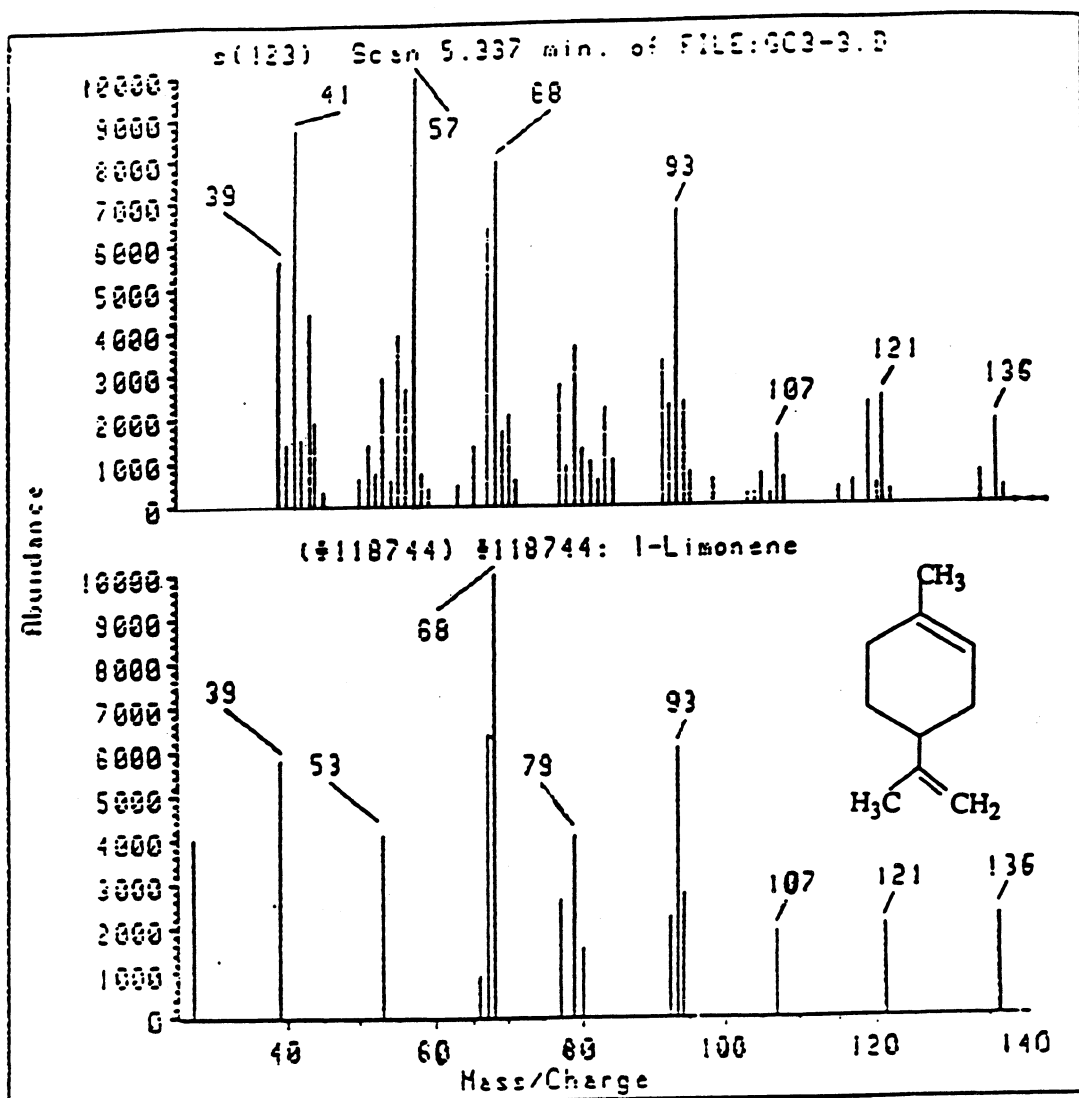


Figure 36: Library match of compound detected in base/neutral methylene chloride extract of untreated Navy 106 dye washwater to limonene.

cyclohexenols, and cyclohexadienes. Terpinene, terpineol, limonene, and several other of these compounds are probably derived from pine oil added to Navy 106 slack wash as a surfactant (Woodby,1992). Tributylphosphoric acid is also added to the slack wash as a surfactant (Woodby, 1992).

Anaerobically Treated Navy 106

Navy 106 washwater was subjected to anaerobic biological treatment by diluting it with 23% Blacksburg municipal primary effluent and 8% anaerobic seed, then digesting this mixture for 15 days at 22 degrees celsius (Figure 11) (Loyd, 1992). Loyd found that the anaerobic treatment scheme described caused a 90% reduction in American Dye Manufacturers Institute (ADMI) color value of the Navy 106 washwater, going from 9584 ADMI units to 5223 ADMI units after dilution with Blacksburg primary effluent and anaerobic seed, and down to 980 ADMI after 15 days of anaerobic treatment. However, this anaerobic treatment caused only a four percent decrease in TOC of the Navy 106. Analyzing this treated washwater by LC/DAD revealed that no detectable dye remained after anaerobic digestion (Figure 37). A 31% reduction in dye concentration would have been effected by the dilution steps even if no dye had been destroyed, but the fact that the post-treatment dye concentrations were below detection limits indicated that dye

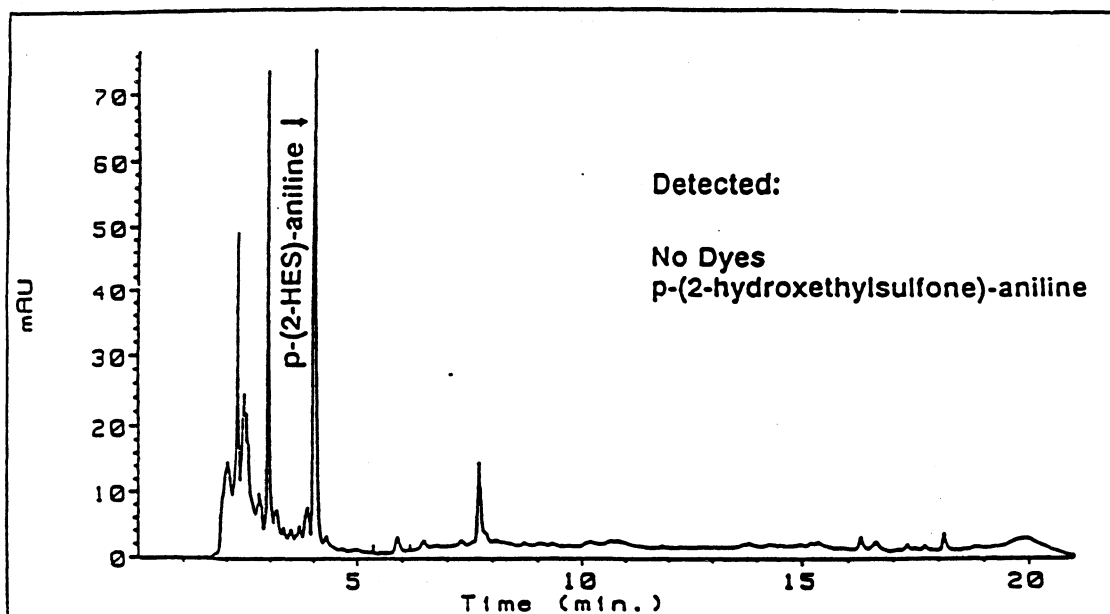


Figure 37: LC chromatogram of anaerobically treated Navy 106 dye washwater on Alltech C18 reverse phase parameters, using a detection wavelength of 254 nm. No dyes were detected. The peak at 4.0 minutes corresponds to p-(2-hydroxyethylsulfone)-aniline, a reduction product of all three dyes of interest.

was removed from the system. For example, if the concentration of hydrolyzed Remazol Black B in undiluted, untreated Navy 106 Dye Washwater was 40 mg/L, a typical value measured for the washwater, dilution would have brought the concentration down to 27.6 mg/L, still above the detection limit of 8.0 mg/L.

p-(2-Hydroxyethylsulfone)-aniline was present in the anaerobically-treated washwater, which is a reduction product of all three dyes of interest. No other reduction products previously identified in laboratory-prepared solutions were found by LC in the anaerobically treated Navy 106. The sulfonated reduction products seen in chemically reduced dye from the manufacturer were apparently not formed or not stable in anaerobically treated Navy 106 Washwater. While chemical reduction probably cleaved the azo bonds and formed two aromatic amines, biochemical reduction may have formed an amine and a nitro or nitroso compound. Alternately, the amines may form but undergo a subsequent redox or rearrangement reaction.

GC/MS of anaerobically treated Navy 106 (Figure 38) confirmed the presence of p-(2-hydroxyethylsulfone)-aniline, and also revealed that many of the products previously identified in untreated Navy 106 survived anaerobic

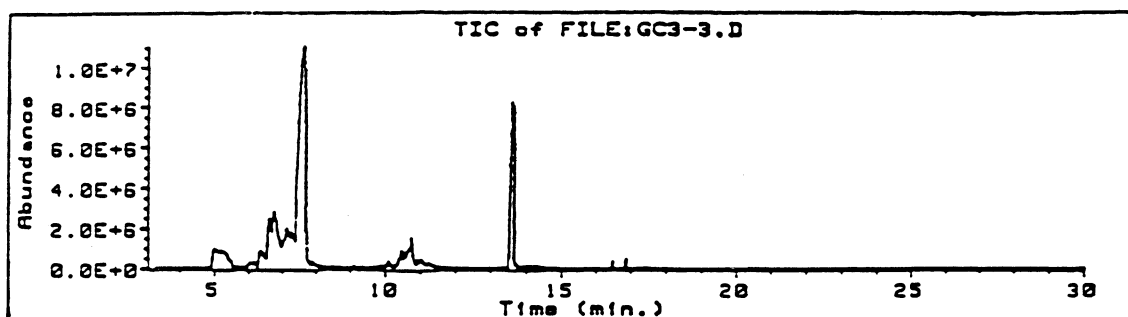


Figure 38: GC/EI-MS chromatogram of base/neutrals methylene chloride extract of anaerobically treated Navy 106 washwater, showing showing little change from untreated Navy 106.

treatment. Other major products tentatively identified included: 1- α -terpineol, endo-fenchol, tributylphosphoric acid, and cyclohexadiene, and limonene.

Anaerobically/Aerobically Treated Navy 106

Loyd (1992) reported that anaerobic treatment followed by aerobic treatment actually led to a significant 2% increase in ADMI color value, but reduced TOC by 47% from the original, not including the effect of dilution. Anaerobic/aerobic treatment significantly reduced the toxicity of Navy 106, resulting in an LD₅₀ of 56% in tests on *Ceriodaphnia dubia*. Analysis by LC/DAD and GC/MS of the effluent both indicated that no dyes were present in solution, and previously identified compounds were not detected after treatment, including 1- α -terpineol, endo-fenchol, tributylphosphoric acid, limonene and substituted cyclohexanols, cyclohexenols, cyclohexadienes and the dye reduction product p-(2-hydroxyethylsulfone)-aniline.

Implications of Results

This research, in conjunction with the results of Loyd, agrees with work performed by other researchers which indicated that anaerobic treatment cleaves the azo bonds and

reduces color but does not significantly reduce TOC of textile waste, while aerobic treatment reduces TOC but does not degrade sulfonated azo dyes (Horning, 1977, Brown and Hamburger, 1987, Brown, 1987, Richardson, 1988, Loyd, 1992).

Liquid chromatography with diode array detection provided an effective and relatively quick method for detecting and quantifying sulfonated azo dyes in aqueous samples. Direct detection limits were calculated to be between one and ten parts per million, but detection of dyes in dilute samples could be greatly improved through the use of pre-concentration steps such as freeze-drying or solid phase extraction. Both reverse-phase and ion-pair chromatography achieved the desired separation of parent, vinyl sulfone, and hydrolyzed forms of the three dyes in Navy 106, though the reverse-phase program was faster and has a more stable baseline.

LC/DAD of fresh Navy 106 indicated that the dyes in the washwater were predominantly in the hydrolyzed form, at concentrations between 4 and 40 mg/L Remazol Black B and between 2 and 37 mg/L Remazol Red RB. The LC/DAD evidence suggests that most of these dyes occur in the washwater because the hydrolysis reaction of the dye successfully competes with dye fixation to cotton. Reducing the rate of

hydrolysis, without greatly reducing the rate of dye fixation, would save dye and reduce the color of the washwater.

Dye reduction products were also monitored in aqueous samples by LC/DAD, but only one reduction product from the chemically reduced dye solutions, p-(2-hydroxyethylsulfone)-aniline, was stable enough and at sufficient concentration to be detected by LC/DAD in anaerobically treated washwater. The presence of this product and the absence of detectable quantities of dye in anaerobically treated Navy 106 washwater, as evidenced by LC/DAD, clearly showed the success of this treatment method in destroying the dyes by cleaving the azo bond and reducing the ADMI color value. In fact, destruction of the dye and the formation of p-(2-hydroxyethylsulfone)-aniline occurred in Navy 106 left in storage for several weeks even without the addition of anaerobic seed, and probably occurs in sewer lines leading from the textile mill to the Martinsville wastewater treatment plant. It is unknown whether this reduction is chemical in nature, biochemical, or both.

If the same reductive derivatives of the sulfonated, naphthalene-based parts of the dyes are forming during anaerobic treatment, they are not stable enough to be detected by LC/DAD and thus probably exist in some other form than the

simple amine expected from reductive cleavage of the azo bond (Riefe, 1992). However, the conjugated orbital system of these compounds gives them the potential to give color to aqueous solutions. Oxidation reactions following reductive cleavage, such as the conversion of an amino group to a nitro or nitroso group, would extend the conjugation of the system and may result in blue, purple, or green colors. During this research, such colors have been observed in anaerobically treated Navy 106, and Navy 106 from storage which exhibited no known dye peaks on LC/DAD. The sulfonic acid groups on these compounds may make them difficult to degrade, resulting in some residual color of the washwater even after anaerobic/aerobic treatment. Meeting stricter color standards in the future may rest on understanding the chemistry of the sulfonated naphthalene-based reductive derivatives of azo dyes, rather than that of the azo bond itself which seems relatively easily broken under anaerobic conditions.

The LC/DAD methods described in this report could be used to monitor for sulfonated azo dyes in environmental samples. For most aqueous samples, such an application would require a pre-concentration step, such as solid phase extraction onto a strong anion exchanger, in order to attain concentrations of dye above the detection limits.

CHAPTER V

CONCLUSIONS

Methods were developed to separate, detect, and quantify three reactive azo dyes using reverse phase and ion-pair liquid chromatography. A reverse phase gradient using 20 mmol pH 5.0 acetate buffer and methanol was successful in separating the three different forms (parent, vinyl sulfone, and hydrolyzed) of each dye with less than twenty minute analysis time. While detection limits using this method were between 1 and 20 mg/L, detection of dyes in very dilute solutions could be accomplished by using a pre-concentration step such as solid phase extraction or freeze-drying. Electrospray LC/MS was used to confirm the hydrolyzed structure of Remazol Black B, and gave clues to the structure of hydrolyzed Remazol Red RB. Both positive- and negative-ion modes gave strong signals, though the negative-ion mode proved superior for analysis of hydrolyzed Remazol Red RB. Thermospray LC/MS did not provide strong enough signals to be of use.

All three dyes of interest yielded two major reduction products when reacted with sodium hydrosulfite. Liquid chromatography of reduced dye solutions was best performed

using an ion-pair gradient of tetrabutylammonium hydroxide, 20 mmol pH 5.0 acetate buffer, and methanol, which required a lengthy analysis time but was able to separate very polar reduction products. One of the reductions products which all three hydrolyzed dyes have in common is p-(2-hydroxyethylsulfone)-aniline, the structure of which was confirmed by both electron impact GC/MS and positive-ion electrospray LC/MS. No mass spectra were obtained of the other reduction products, which are probably sulfonated derivatives of the larger, naphthalene-based chromophores of the dyes. These products were also probably responsible for the residual color of dye solutions after chemical reduction.

Analysis of treated and untreated Navy 106 Dye Washwater was performed. All three dyes of interest were detected in untreated Navy 106 washwater; Remazol Black B was detected in it's hydrolyzed form. GC/MS of untreated Navy 106 revealed the presence of over thirty organic compounds such as tributylphosphate, alpha-terpineol, limonene, and endo-fenchol. LC/DAD and GC/MS analysis of Navy 106 after anaerobic biological treatment revealed the absence of all three dyes and the presence of p-(2-hydroxyethylsulfone)-aniline, a known reduction product of all three dyes. Anaerobic treatment succeeded in reducing the ADMI color value

of Navy 106 Dye Washwater, though most of the organic compounds detected by GC/MS in untreated Navy 106 were also detected in anaerobically treated washwater. Navy 106 after anaerobic/aerobic treatment contained no dye, no known reduction products, and none of the products previously detected by GC/MS. This agrees with the results of other researchers (Brown and Hamburger, 1987, Brown, 1987, Richardson, 1988) which indicate that microbes can cleave azo bonds under anaerobic conditions, but aerobic treatment is required to reduce TOC.

CHAPTER VI

RECOMMENDATIONS

It is recommended that further studies of this nature investigate the fate of the sulfonated reduction products of azo dyes. The structures of the stable sulfonated products of azo dyes remain unknown, and because these compounds are large and polar they may be less susceptible to microbial degradation. Their ability to impart color to solutions even after reduction of the azo bond may make them the controlling factor in meeting extremely low color effluent standards.

Continuing research of the analysis of dyes and their degradation products should concentrate on the use of on-line LC/MS, flow FAB, CZE, or other techniques to elucidate the structures of dye reduction products, especially those which are sulfonated, and their subsequent reaction products. Such work may lead to an understanding of what stable products form under different conditions of pH, redox potential, and solution chemistry.

REFERENCES

- Abeta, S., Kingo, A., Meyer, U., and Zollinger, H., "Cellulose Dyeing Parameters of 18 Isomeric Phenylazo-H-acid Dyes Containing Two Different Reactive Groups." *Jour. of the Soc. of Dye Chemists* 107, p. 12-19, (1991).
- Abraham, E. N., *Dyes and Their Intermediates*. Pergamon Press, Oxford, 335 p., (1968).
- Ballard, J. M., and Betowski, L. D., "Thermospray Ionization and Tandem Mass Spectrometry of Dyes." *Organ. Mass. Spec.* 21, p. 575-588, (1986).
- Barber, M., Bordoli, R. S., Sedgwick, R. D., and Tyler, A. N., "Fast Atom Bombardment of Solids as an Ion Source in Mass Spectrometry." *Nature* 293, p. 270-275, (1981).
- Baughman, George L., and Perenich, Theresa A., "Fate of Dyes in Aquatic Systems: Solubility and Partitioning of Some Hydrophobic Dyes and Related Compounds." *Env. Tox. and Chem.* 7, p. 183-199 (1988).
- Beech, W. F., *Fibre-Reactive Dyes*. Logos Press LTD., London, 400 p., (1970).
- Behymer, T. D., Bellar, T. A., and Budde, W. L., "Liquid Chromatography/Particle Beam/Mass Spectrometry of Polar Compounds of Environmental Interest." *Anal. Chem.* 62, p. 1686-1690, (1990).
- Bellar, Thomas A. and Budde, William L., "Determination of Nonvolatile Organic Compounds in Aqueous Environmental Samples Using Liquid Chromatography/Mass Spectrometry" *Anal. Chem.* 60, p. 2076-2083, (1988).
- Betowski, Leon D. and Ballard, John M., "Identification of Dyes by Thermospray Ionization and Mass Spectrometry/Mass Spectrometry" *Anal. Chem.* 56, p. 2604-2607, (1984).
- Betowski, Leon D. and Ballard, John M., "Thermospray Ionization and Tandem Mass Spectrometry of Dyes" *Organic Mass. Spec.* 21, p. 575-588, (1986).

- Betowski, L. D., Pyle, S. M., Ballard, J. M., and Shaul, G. M., "Thermospray LC/MS/MS Analysis of Wastewater for Disperse Azo Dyes." *Biomed. and Env. Mass Spec.* 14, p. 343-354, (1987).
- Brown, D., Hitz, H. R., and Schafer, L., "The Assessment of the Possible Inhibitory Effect of Dyestuffs on Aerobic Waste-Water Bacteria: Experience With a Screening Test.", *Env. Tox. and Chem.* 1 p. 245-258, (1981).
- Brown, D. and Laboureur, P., "The Degradation of Dyestuffs, Part I: Primary Biodegradation Under Anaerobic Conditions." *Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers (ETAD)*, Clarastrasse 4, Basel 5 CH-4005, Switzerland (1987).
- Brown, D. and Laboureur, P., "The Degradation of Dyestuffs, Part II: The Aerobic Biodegradability of Primary Aromatic Amines." *Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers (ETAD)*, Clarastrasse 4, Basel 5 CH-4005, Switzerland (1987).
- Bruins, A. P., Weidolf, L. O. G., and Henion, J. D., "Determination of Sulfonated Azo Dyes by Liquid Chromatography/Atmospheric Pressure Ionization Mass Spectrometry" *Anal. Chem.* 59, p. 2647-2652, (1987).
- Camp, S. K. R., *The HPLC Separation and Electrochemical Detection of Azo Dyes and Their Degradation Products.* Doctoral dissertation, Dept. of Chemistry, Ga. Instit. Tech., (1989).
- Dabney, Betty J., DeBroy, James A., Freeman, H. S., Price, K. B., Esancy, J., Calogero, F., Kennedy, K. M., and Whaley, W. M., "Structure-Activity Relationships for Azo Dyes Made From Aminonaphthalenes." Submitted to Office of Toxic Substances, U. S. E. P. A., (1983).
- Danzig, A. J., *Chromatographic and Mass Spectrometric Characterization of a Landfill Leachate and an Industrial Wastewater.* Master's thesis, Dept. of Civil Eng., Virginia Polytechnic Institute and State University, (1990).

- Eberhard, J. S., Giolando, S. T., and Tabor, M. W., "Biotic and Abiotic Degradation of Azo Dyes in Aerobic Bioreactors." Preprint Extended Abstract, Amer. Chem. Soc. Div. of Env. Chem., p. 19-20 (1990).
- Fischer, C. H., Bischof, M., and Rabe, J. G., "Identification of Natural and Early Synthetic Textile Dyes with HPLC and UV/Vis-Spectroscopy by Diode Array Detection." *Jour. Liquid. Chrom.* 13 (2), p. 319-331, (1990).
- Flory, Donald A., McLean, Matthew M., Vestal, Marvin L., and Betowski, Leon D., "Environmental Application fo Thermospray LCMS: Qualitative Analysis of Sulfonated Azo Dyes." *Rapid Comm. in Mass. Spec.* 1 (3), p. 48-50, (1987).
- Freeman, Harold S., Whaley, Wilson M., Esancy, Michelle K., and Esancy, James F., "Anomalous Behavior of Aminohydroxynaphthalenesulfonic Acids During Diazo Coupling." *Dyes and Pigments* 7, p. 215-230 (1986).
- Freeman, H. S., Sokolowski-Gajda, J., and Hao, Z., "Analysis of Some Azo and Anthraquinone Dyes." *Textile Chem. and Color.* 21 (4), p. 25-29, (1989).
- Games, Larry M., and Hites, Ronald A., "Composition, Treatment Efficiency, and Environmental Significance of Dye Manufacturing Plant Effluents." *Analytical Chemistry* 49 (8), p. 1433-1440, (1977).
- Ganesh, R. *Fate of Azo Dyes in Sludges*. Master's thesis, Dept. of Civil Eng., Virginia Polytechnic Institute and State University, (1992).
- Gardner, David A., Holdsworth, Thomas J., Shaul, Glenn M., Dostal, Kenneth A., and Betowski, Don L., "Aerobic and Anaerobic Treatment of C. I. Disperse Blue 79." U. S. E. P. A. Project Summary, Risk Reduction Engineering Lab., 6 p., (1990).
- Gilland, J. C. Jr. and Lewis, J. S., "Mass Spectrometry of 4-Amino-4'-Nitroazobenzene Compounds." *Organ. Mass. Spec.* 9, p. 1148-1153, (1974).

- Giolando, S. T., Williams, J. J., and Tabor, M. W., "Solid Phase Extraction-HPLC Separation/Quantitation of Dyestuffs in Wastewaters." Preprint Extended Abstract, Amer. Chem. Soc. Div. of Env. Chem., p. 17-18, (1990).
- Loyd, C. Kemper, *Anaerobic/Aerobic Degradation of a Textile Dye Wastewater*. Master's thesis, Dept. of Civil Eng., Virginia Polytechnic Institute and State University (1992).
- McLean, Matthew A., and Freas, Royal B., "Enhanced Analysis of Sulfonated Azo Dyes Using Liquid Chromatography/Thermospray Mass Spectrometry." *Anal. Chem.* 61, p. 2054-2058, (1989).
- McMurry, J., *Organic Chemistry*. Coleman/Brooks Publishers, New York, NY, 672 p., (1988).
- Miller, James B., *Chromatography: Concepts and Contrasts*. Wiley-Interscience, New York, 297 p., (1988).
- Pagga, U., and Brown, D., "The Degradation of Dyestuffs: Part II Behaviour of Dyestuffs in Aerobic Biodegradation Tests." *Chemosphere* 15 (4), p. 479-491, (1986).
- Parry, W., Research Chemist, Hoechst-Celanese Corp., pers. comm. (1992).
- Riefe, A., private consultant, pers. comm. (1992).
- Singer, B. and Grunberger, D., *Molecular Biology of Mutagens and Carcinogens*. Plenum Press. New York, 1983.
- Sturrock, P. E., *The Analysis of Azo Dyes in Water by High-Performance Liquid Chromatography With a Swept-Potential Electrochemical Detector*. Technical Completion Report for Project USGS G1219 (06), 17 p., (1987).
- Voyksner, Robert D., "Characterization of Dyes in Environmental Samples by Thermospray High-Performance Liquid Chromatography/Mass Spectrometry" *Anal. Chem.* 57, p. 2600-2605, (1985).

- Voyksner, R. D., Straub, R., and Keever, J. T., "Determination of aromatic amines originating from azo dyes by chemical reduction combined with liquid chromatography/mass spectrometry." *Environ. Sci. & Tech.* 27 (8), p. 1665-1672, (1993).
- Watson, J. Throck, *Introduction to Mass Spectrometry*. Raven Press, New York, 351 p. (1985).
- Weber, Eric J., Sturrock, Peter E., and Camp, Sharon R., "Reactive Dyes in the Aquatic Environment: A Case Study of Reactive Blue 19." U. S. E. P. A. Environmental Research Brief, 7 p., (1990).
- White, Peter C., and Catterick, Timothy, "Use of Colour Coordinates and Peak Purity Parameters for Improving the High-performance Liquid Chromatographic Qualitative Analysis of Dyes." *Analyst* 115, p. 919-923, (1990).
- Woodby, Rebecca D., Research Assistant, Dept. of Chem. Eng., VPI &SU, pers. comm. (1992).
- Yergey, A. L., Edmonds, C. G., Lewis, I. A. S., and Vestal, M. L., *Liquid Chromatography/Mass Spectrometry*. Plenum Pub. Co., New York, 306 p., (1990).
- Yinon, J., Jones, T. L., and Betowski, L. D., "Enhanced Sensitivity in Liquid Chromatography/Thermospray Mass Spectrometry Using a Wire Repeller." *Rapid Comm. in Mass Spec.* 3 (2), p. 38-41, (1989).
- Yinon, J., Jones, T. L., and Betowski, L. D., "High-sensitivity Thermospray Ionization Mass Spectrometry of Dyes." *Biomed. and Env. Mass Spec.* 18, p. 445-449, (1989).
- Yinon, J., Jones, T. L., and Betowski, L. D., "Particle Beam Liquid Chromatography-Electron Impact Mass Spectrometry of Dyes." *Jour. Chrom.* 482, p. 75-85, (1989).
- Zollinger, Heinrich, *Azo and Diazo Chemistry: Aliphatic and Aromatic Compounds*. Interscience Pub., New York, NY, 712 p., (1961).

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