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A SPECTROSCOPIC STUDY OF RHODAMINE-6G: DETECTION OF NONABSORBING ANALYTES IN REVERSE-PHASE CHROMATOGRAPHY WITH THE AID OF RHODAMINE-6G

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A SPECTROSCOPIC STUDY OF RHODAMINE-6G: DETECTION OF NONABSORBING ANALYTES IN REVERSE-PHASE CHROMATOGRAPHY WITH THE AID OF RHODAMINE-6G

by

Panayotis Dimitrios Sokrates Tsakanikas

A Thesis Submitted to the Faculty of the

DEPARTMENT OF CHEMISTRY

In Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 8 5

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Date

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ABSTRACT

In order to understand the mechanism by which dyes assist chromatographic detection of diverse solutes in reverse phase systems, absorption and fluorescence spectra of rhodamine-6G were investigated. It is shown that rhodamine-6G dimerizes in water-methanol solutions of different composition. Aggregation increases as the concentration of the dye increases and the methanol content of the solvent decreases. Contrary to dimers, monomers exhibit strong absorption and fluorescence. Detection of analytes separated in a chromatographic column is based on their ability to break up dimers.

The fluorescence of rhodamine-6G was used to detect aliphatic alcohol reverse phase chromatography. The results of these experiments are reported.

CHAPTER 1

INTRODUCTION

The physical and chemical properties of compounds upon which analytical methods are based are rarely specific to a particular compound. Many compounds exhibit similar or even identical properties. Therefore it is essential to eliminate interferences before a chemical analysis is carried out. Interferences are eliminated very often by separating the analyte from a complex mixture. Unquestionably the most popular separation techniques involve chromatographic methods.

In the chromatographic method, a mobile phase carries a mixture of components through a stationary phase and separation occurs due to the differences in migration rates between the components of the sample. When a nonpolar stationary phase and a polar mobile phase are employed, the technique is called reverse phase chromatography.

Figure 1 is a schematic diagram showing liquid flow in a typical reverse phase chromatographic system.[1] Solvent from two glass reservoirs is mixed in a mixing vessel as elution begins, and by means of a high pressure pump the solvent is pumped through a precision-bore stainless steel column of variable length and diameter. The sample is introduced into the rotary valve of an injection port by a syringe, and a slider is then switched to force the sample into the solvent stream. Separation occurs in the column and as the separated components leave the column, they are detected.

Depending on the particular properties of the sample components

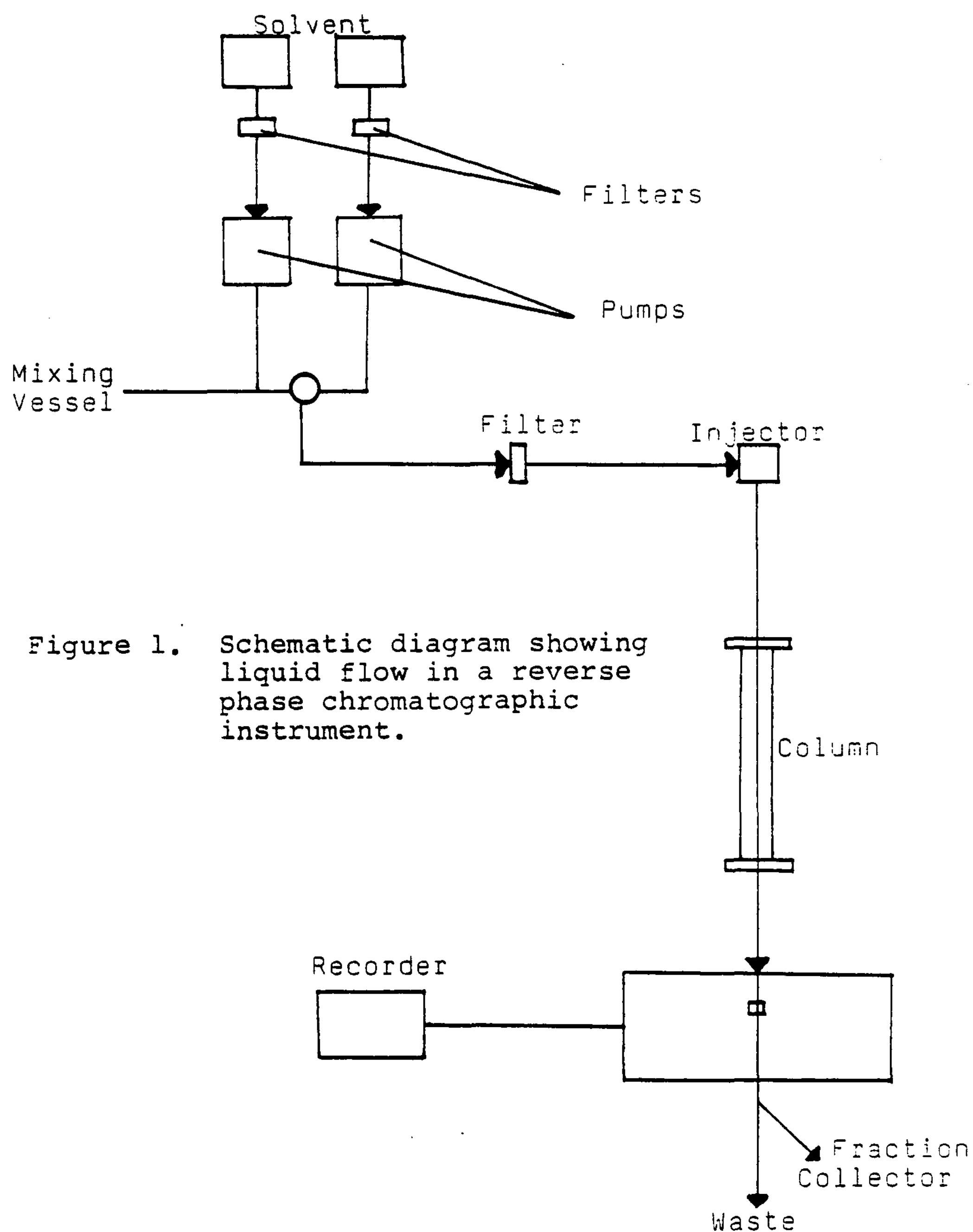


Figure 1. Schematic diagram showing liquid flow in a reverse phase chromatographic instrument.

that are separated by reverse phase chromatography, various detector systems are employed.

The most common detectors are based upon absorption of visible or ultraviolet radiation and require that the sample components absorb and the solvent transmit radiation at the wavelength of detection.

Detection that is based upon differences in refractive index between the solvent and analyte solutions is currently used for compounds that do not absorb in the uv-visible region of the spectrum or absorb at widely different wavelengths. Here the two solutions are separated by a glass plate adjusted at an angle such that the incident beam bends when there is a difference in refractive index. Other detectors utilize absorption of infrared radiation, fluorescence, mass spectroscopy or polarography. However, no universal detector system such as the one used in gas chromatography is available for reverse phase chromatography.

Absorption or fluorescence of dyes, added as probe molecules to the mobile phase can provide a common method of detection for diverse solutes.

Recent studies of methylene blue and brilliant green have shown that analytes separated in a chromatographic column, enhance the absorbance of the probe molecules and thus all of them can be detected at one wavelength, namely at the dye absorption peak.[3,4] This method promises higher sensitivity over the refractive index method. In spite of this, chromatographic studies with other dyes have not yet been undertaken. This is undoubtedly because the detailed steps by which dyes assist chromatographic detection have not yet been completely

explored. It is only recently that rudiments of a mechanism for dye action has been suggested.[2] According to the proposed mechanism, dyes dimerize appreciably in aqueous-methanolic solutions at the concentrations used in reverse phase chromatographic experiments. Dimers exhibit hypochromism presumably due to cancellation of transition dipole moments. Solutes shift the monomer-dimer equilibrium of the dyes toward greater monomer concentration and thus enhance their absorbance.

For many cases fluorescence provides a better means of detection and greater sensitivity compared to absorption. This is due to the fact that low level fluorescence can be readily detected with minimal background noise. It has been known for some time that the monomeric form of dyes fluoresces intensely and the dimeric form fluoresces weakly, or not at all.[5] Since solutes promote the breakup of dimers, we should be able to detect them through an increase in the fluorescence intensity of the dye. No dye, however, has been studied in this context for understanding its potential as a chromatographic probe. Methylene blue does not exhibit appreciable fluorescence and brilliant green fluoresces in the infrared region of the electromagnetic spectrum where fluorescence has poor quantum yields.

A strongly absorbing and highly fluorescent dye, in the uv-visible region of the spectrum, must be studied in order to ascertain the potential of fluorescence at one wavelength as an indicator of concentrations of diverse solutes. In addition, the same dye can be used to establish whether the applicability of the absorption detection technique is limited to methylene blue and brilliant green or extends to many other dyes that are available. We chose rhodamine-6G for such an

investigation, because it satisfies these criteria; it exhibits strong absorption and fluorescence in the visible region of the electromagnetic spectrum. Besides this, there is another important reason for this choice. Rhodamine-6G is a commonly used laser dye. The laser action of this dye depends on having large monomer concentrations. If solutes break the dimers and enhance the fluorescence of the dye, they should drastically alter the lasing properties of the latter. This effect could be used in intercavity detection of solutes.

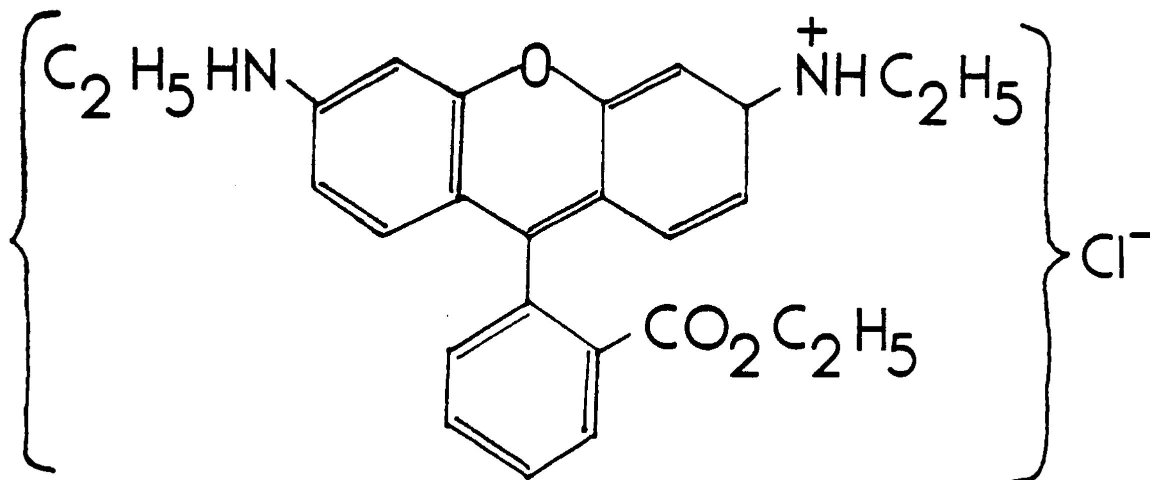
A comprehensive study of the properties of rhodamine-6G in relation to chromatography should include three phases. First, the absorption spectra of the dye in varied composition water-methanol or water-ethanol solvent systems should be obtained and analyzed to determine solvent effects on dimerization and concentration dependence of the absorbance. The solutions must cover a concentration range commonly used in chromatographic experiments. Second, the fluorescence of these solutions should be examined as a function of concentration and solvent composition. Third, chromatographic separations of analytes should be investigated using both dye absorption and dye fluorescence detection in order to evaluate the usefulness of these techniques.

In this work, we present a study of the absorption and fluorescence spectra of rhodamine-6G in varied composition water-methanol solutions. The concentrations we investigated range from 10^{-4}M to 10^{-6}M. We used the fluorescence of this dye to detect aliphatic alcohols separated by reverse phase chromatography. The results of these preliminary experiments are also described in this report.

CHAPTER 2

EXPERIMENTAL

Analytical grade rhodamine-6G was obtained from Eastman Kodak Chemical Company and used without further purification.



ACS certified reagent grade methanol and double distilled water were used as the primary solvents at percentage compositions ranging from 100% methanol to 100% water.

Dye solutions of concentrations varying from 10^{-4}M to 10^{-6}M were prepared by diluting 10^{-3}M stock solutions.

Absorption spectra were recorded with the aid of a Perkin Elmer 552 uv-visible spectrophotometer by using absorption cells of path lengths 0.1cm and 1.0cm.

For right-angle fluorescence measurements we used a Perkin Elmer 550 spectrophotofluorometer that employed a 150 Xenon power supply. The filters used, allowed transmission of 6.3% of the light through the excitation slit and 6.0% through the emission slit, resulting in a net light transmission of 0.4%.

Front surface fluorescence measurements were made in a similar manner for each solution, by using a Spexflurolog spectrophotometer.

For the reverse phase chromatography experiments we used a Beckman high performance liquid chromatograph that consisted of a 420 controller with a C-18, 25cm column, two 110B pumps and a Kratos fluorescence detector. The mobile phase was 5.2×10^{-4}M solution of rhodamine-6G in 90% water and 10% methanol by volume. Mixtures of varying composition of ACS certified reagent grade 1-propanol, 1-butenol, 1-pentanol were studied at 1/10, 1/100, 1/1000 ratios of solute to solvent.

All experiments were performed at the ambient laboratory temperature (24°C).

CHAPTER 3

ABSORPTION STUDIES OF RHODAMINE-6G

Absorption of visible radiation by matter involves excitation by absorption of a photon, hv, as shown by the equation:

where A* represents the molecule in its electronically excited state. This state has in general a very short lifetime (10^{-8} - 10^{-9}s), and is terminated by radiative or nonradiative relaxation processes.

One type of relaxation results in conversion of excitation energy to heat;

Other processes involve emission of fluorescent or phosphorescent radiation, or decomposition of A*.

In absorption spectroscopy two terms are defined to describe the interactions between the photons and absorbing species: transmittance and absorbance.

Transmittance T of the solution is the fraction of incident radiation transmitted by the solution, and is frequently expressed as a percentage:

$$T = \frac{P}{P_o}$$

where P_o = power of incident beam

P = power of emerging beam

The Absorbance of the solution is defined by the equation:

$$A = -\text{Log}_{10}\, T$$

and is directly proportional to the concentration of the absorbing species and the path length through the solution. That is,

$$A = abc$$

where a = molar absorptivity in liters/mole x cm

b = cell length in cm

c = concentration in moles/liter.

This is a statement of Beer's Law.

The absorption of visible light is the result of excitation of bonding electrons, and therefore absorption peaks can be correlated with specific structural characteristics of the species under study.

The absorption spectra of a series of 10^{-4}M and 10^{-5}M - 10^{-6}M rhodamine-6G solutions of varying water-methanol compositions are shown in Figures 2 and 3 respectively. Each spectrum consists of a relatively broad peak at 525nm and a shoulder at 502nm.

The linear relationship between absorbance and path length when the concentration of the solution remains unchanged is a fact, and exceptions are not known. On the other hand, deviations from the direct proportionality between absorbance and concentration, at constant path length are often encountered, and the spectra in Figures 2 and 3 provide one such example. Figure 4 shows the concentration dependence of

FIGURE CAPTION

Figure 2. Absorption spectra of rhodamine-6G. Solvent: 100% water, 90% water - 10% methanol, 80% water - 20% methanol, 50% water - 50% methanol, 100% methanol. Path length: 0.1cm.

Concentration: 2.0×10^{-4}M, 5.2×10^{-4}M.

* - 2.0×10^{-4}M 100% water

5.2×10^{-4}M

+ - 100% water

^ - 90% water, 10% methanol

< - 80% water, 20% methanol

> - 50% water, 50% methanol

x - 100% methanol

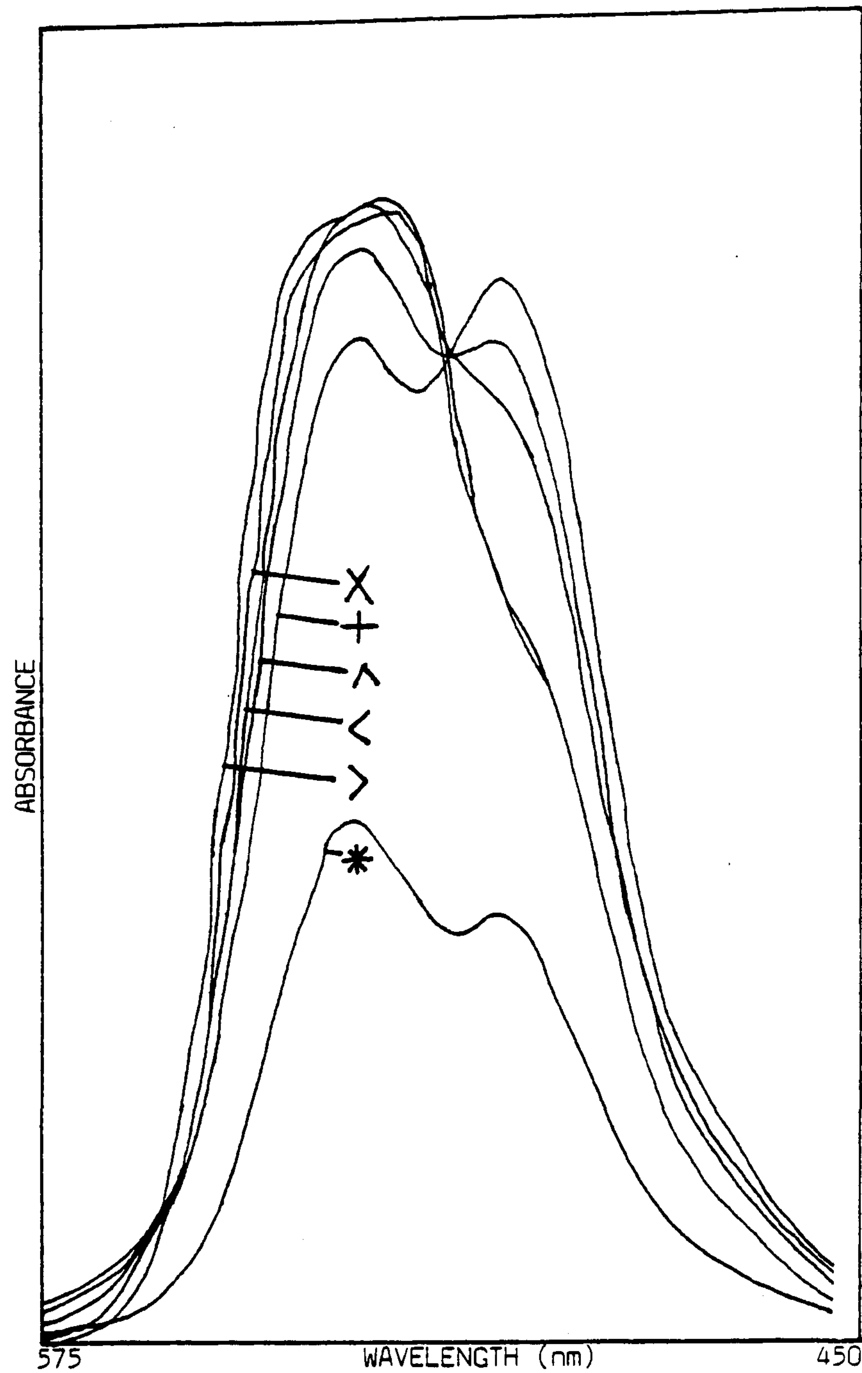


Figure 2. Absorption spectra of rhodamine-6G.

FIGURE CAPTION

Figure 3. Absorption spectra of rhodamine-6G. Solvent: 100% water, 90% water - 10% methanol, 80% water - 20% methanol, 100% methanol. Path length: 1.0cm.

Concentration: 5.2×10^{-6}M, 5.2×10^{-5}M, 2.0×10^{-5}M

* - 5.2×10^{-6}M - 100% methanol

+ - 5.2×10^{-6}M - 100% water

^ - 5.2×10^{-6}M - 90% water, 10% methanol

< - 5.2×10^{-6}M - 80% water, 20% methanol

> - 5.2×10^{-5}M - 100% methanol

x - 5.2×10^{-5}M - 90% water, 10% methanol

** - 5.2×10^{-5}M - 80% water, 20% methanol

++ - 5.2×10^{-5}M - 100% water

^^ - 2.0×10^{-5}M - 100% water

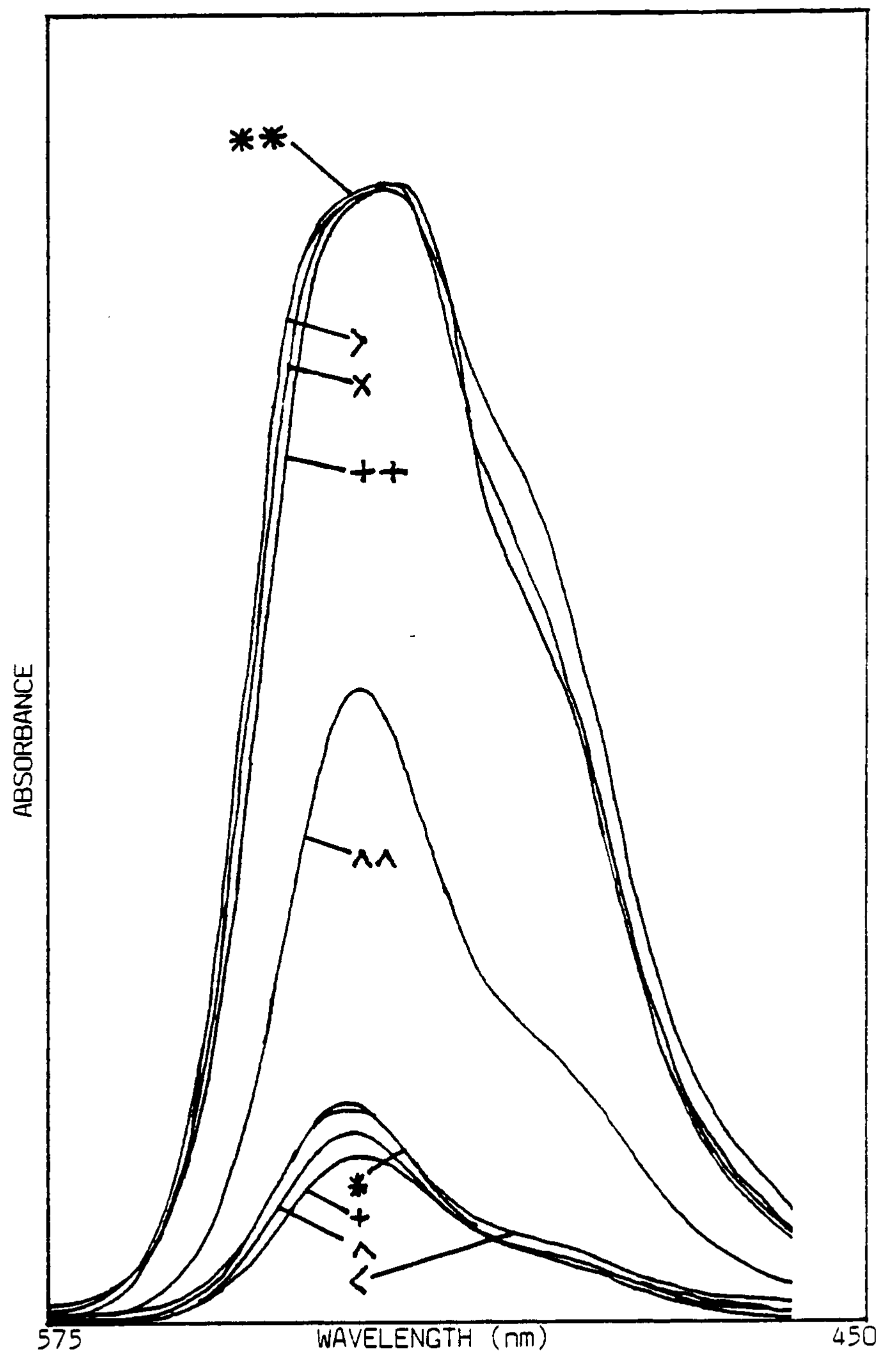


Figure 3. Absorption spectra of rhodamine-6G.

FIGURE CAPTION

Figure 4. Dependence of absorbance on concentration of rhodamine-6G. Solvent: 100% water, 100% methanol.

A - 100% methanol

B - 100% water

o = 100% methanol

Δ = 100% water

Lines A and B denote absorbance predicted from Beer's law. In calculating the points for these lines it is assumed that solutions of 5.2×10^{-6}M consist of monomers only. The points shown are the observed values.

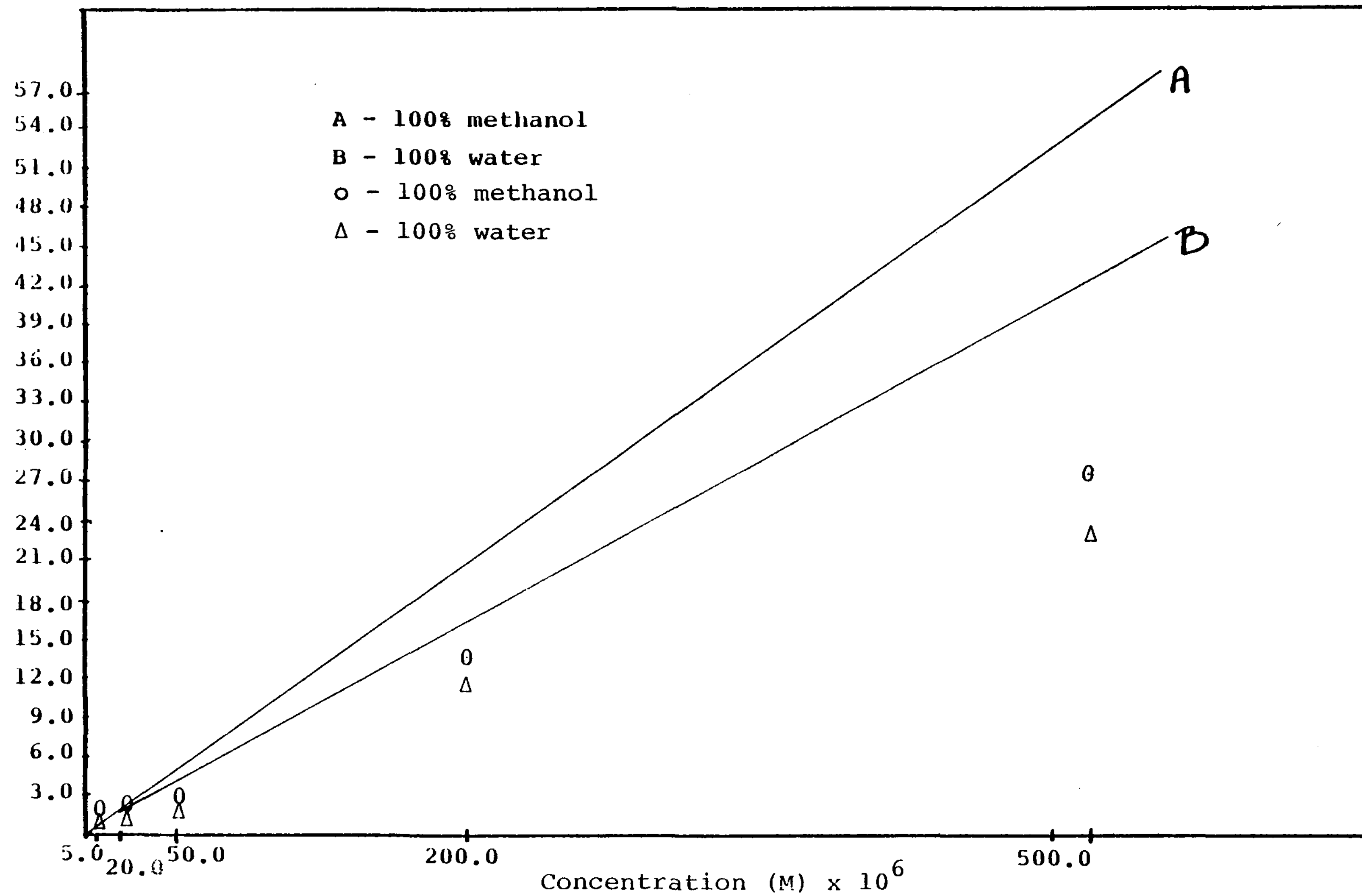


Figure 4. Dependence of absorbance on concentration of rhodamine-6G.

absorbance for the rhodamine-6G systems. As can be readily seen, Beer´s Law is not obeyed and molecular interactions become increasingly evident as the concentration increases.

A number of dyes have been found to undergo association and the association phenomenon has been investigated extensively.[6-9] The apparent deviation from Beer´s Law is considered to be a consequence of dimer formation of the absorbing dye molecules. The absorbance maximum at 525nm is primarily due to the monomer since it persists in dilute solutions. The peak at 502nm is assigned to the dimer form of the dye since it is prominent at higher concentrations. The result of dimerization is the lowering of the absorptivity and a partial splitting of the monomer band into two bands. Dimers have lower absorptivity than monomers due to cancellation of transition dipoles.[5] Therefore, in dye assisted chromatographic detection, the highest possible dye concentration is desired in the mobile phase.

An interesting aspect of these systems is concerned with solvent effects. The total absorbance of the solution depends upon the concentration ratio between the dimer and the monomer. The addition of small amounts of low molecular weight alcohols to aggragated aqueous solutions is expected to break aggregates and thus enhance the absorbance of these solutions. This was found to be the case for methylene blue, cyanines[10], and thionine.[11] As it is shown in Figure 3, this is true for rhodamine-6G as well. An 80% water - 20% methanol solution exhibits a considerably lower degree of dimerization than the aqueous solution of the same concentration. Our results, however, show

evidence of dimer presence even in 100% methanol solution. This is contrary to the Selwyn and Steinfeld[12] claim that rhodamine-6G remains monomeric in alcoholic solutions until concentrations of the order of 10^{-2}M are reached.

Based on this information, we recommend the lowest possible amount of methanol, in the mobile phase. This assures that a large portion of the dye is present in the dimeric form and, therefore, a shift of the monomer-dimer equilibrium by analytes toward higher monomer concentration becomes more apparent.

CHAPTER 4

FLUORESCENCE STUDIES OF RHODAMINE-6G

The underlying phenomena that determine the importance of fluorescence are the result of many compounds being excited by electromagnetic radiation, and as a consequence reemitting radiation of longer wavelength.

When an electron in a molecule is excited to one of the vibrational levels of a higher electronic energy level by absorption of radiation, a singlet or a triplet state results. In the case of a singlet state the spin of the promoted electron continues to be paired with the ground state electron; in the triplet state however, the spins of the two electrons are parallel in orientation. Emission from a high excited singlet state S_2 (Figure 5),[1] is rarely observed, because radiationless processes lead to a transition from that state to the lowest excited singlet state S_1. Three types of transitions may occur from the latter: 1) radiationless intersystem crossing to the triplet state T_1, 2) radiationless internal and external conversion to the ground state, 3) radiative transition to the ground state leading to significant fluorescence.

Thus, fluorescence from a solution involves a transition from the lowest excited state to one of the vibrational levels of the ground state.

A property of a fluorescent molecule is the quantum yield, which is defined as the ratio of the energy emmitted to the

FIGURE CAPTION

Figure 5. Excitation and deactivation by radiative and radiationless transitions. Radiative transitions are indicated by solid lines, and radiationless transitions are indicated by wavy lines. The fine structure of lines indicates schematically vibrational and rotational levels.

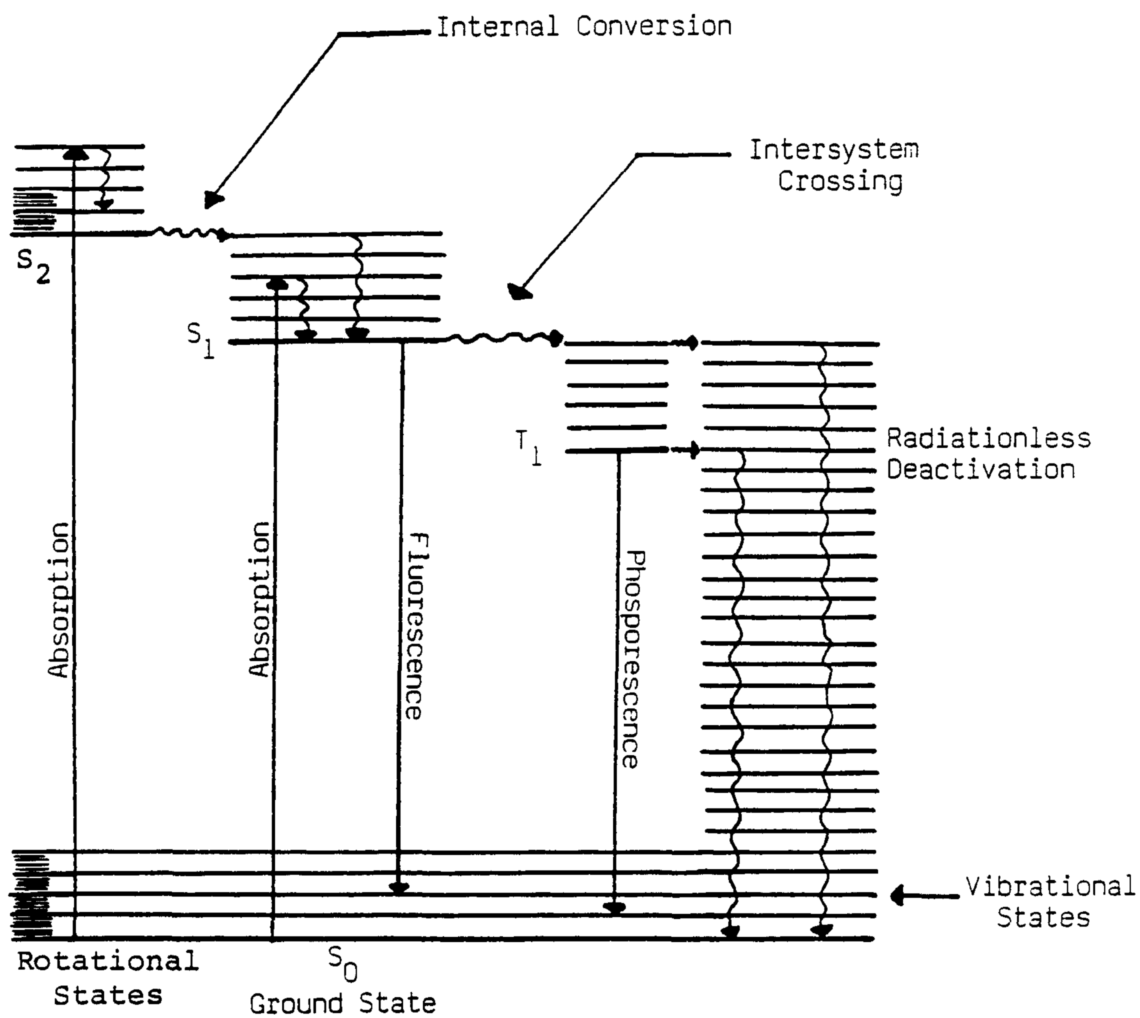


Figure 5. Excitation and deactivation by radiative and radiationless transitions.

energy absorbed. For highly fluorescent compounds the quantum yield may approach unity.

Important factors that influence the quantum yield include pH and the particular structural characteristics of the system under study. In the case of liquid chromatographic applications, for example, the types of solvents used and the water content of the mobile phase play a significant role in quantum yield determination.

In Figure 6 the fluorescence spectra of rhodamine-6G solutions in pure water at different concentrations are shown. These spectra were obtained by excitation at the wavelength corresponding to maximum absorbance of the monomer.

The shapes of the curves of the 10^{-6}M and 10^{-5}M solutions, are structurally similar to those of the absorption curves for the same solution. We see a primary band at 550nm and a shoulder at about 580nm. As the concentration increases, these bands are shifted to longer wavelengths. Further, as the concentration increases, the intensity of the shoulder increases, and at 5.2×10^{-4}M concentration level, we observe one broad band at 590nm. The fluorescence spectra of dye solutions of different water-methanol ratios are shown in Figures 7-9. The shapes of the curves of these solutions exhibit similar structural trends. (For details see figure captions).

It is known that monomers fluoresce intensely and dimers exhibit only weak fluorescence.[5] When the intensity of the fluorescence I_f is plotted against the concentration, as shown in Figure 10, we observe that as the concentration decreases fluorescence increases exponentially. Also the higher the methanol fraction in the solvent,

FIGURE CAPTION

Figure 6. Right angle fluorescence spectra of rhodamine-6G. Solvent: 100% water.

Concentration	Sensitivity Range
x - 5.2 x 10^{-6}M	0.3
+ - 2.0 x 10^{-5}M	0.3
* - 2.0 x 10^{-4}M	3.0
^ - 5.2 x 10^{-4}M	10.0

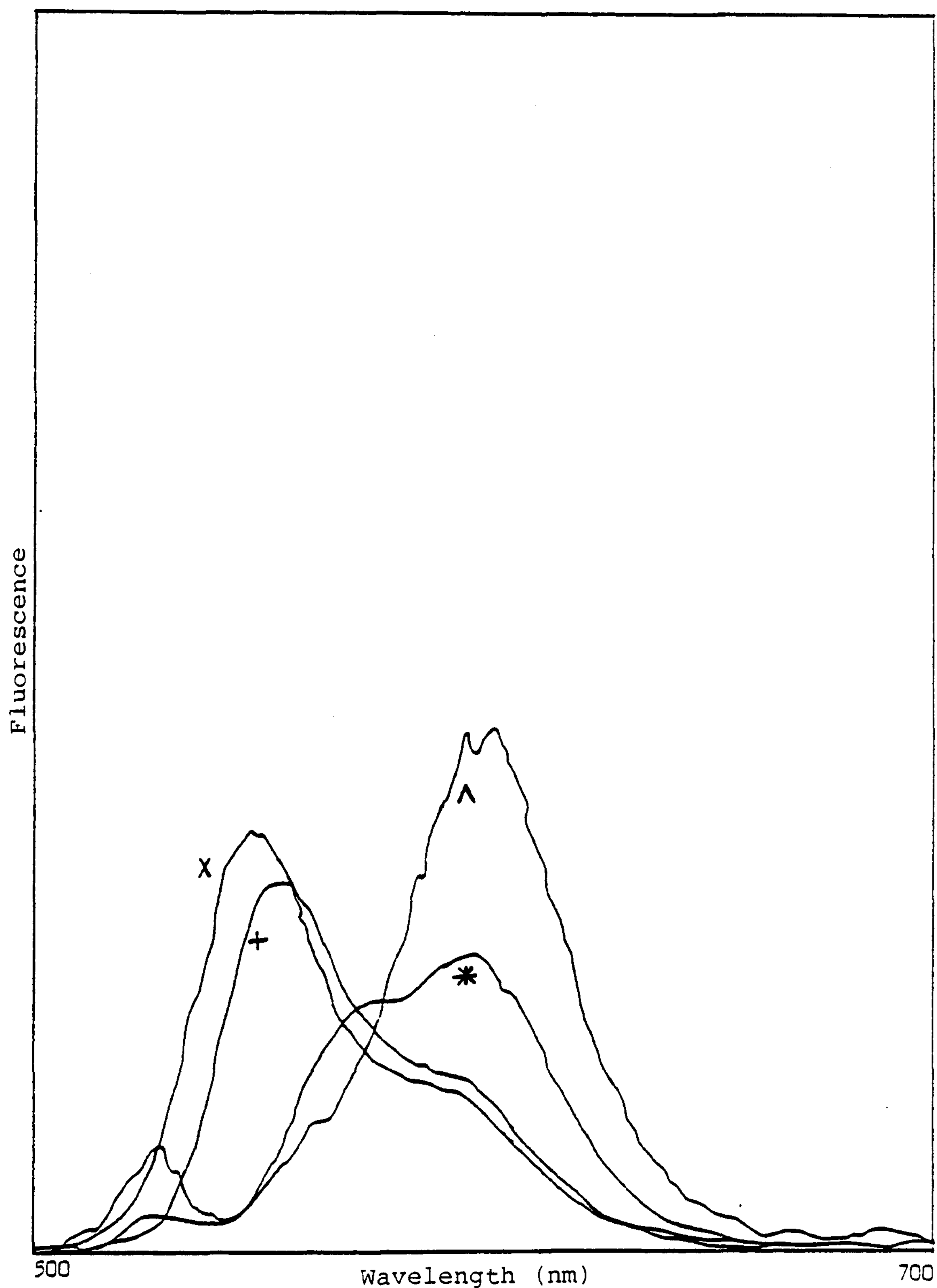


Figure 6. Right angle fluorescence spectra of rhodamine-6G.

FIGURE CAPTION

Figure 7. Right angle fluorescence spectra of rhodamine-6G. Solvent: 90% water, 10% methanol.

Concentration	Sensitivity Range
x - 5.2×10^{-6}M	0.3
+ - 5.2×10^{-5}M	1.0
* - 5.2×10^{-4}M	10.0

COMMENT PERTAINING TO FIGURES 7-9: The curves corresponding to the 5.2×10^{-6}M and 5.2×10^{-5}M solutions of the dye exhibit a mirror image relation with the absorption curves of the same solutions. The 5.2×10^{-4}M spectra consist of one broad band. The curves are shifted to longer wavelengths as the concentration increases.

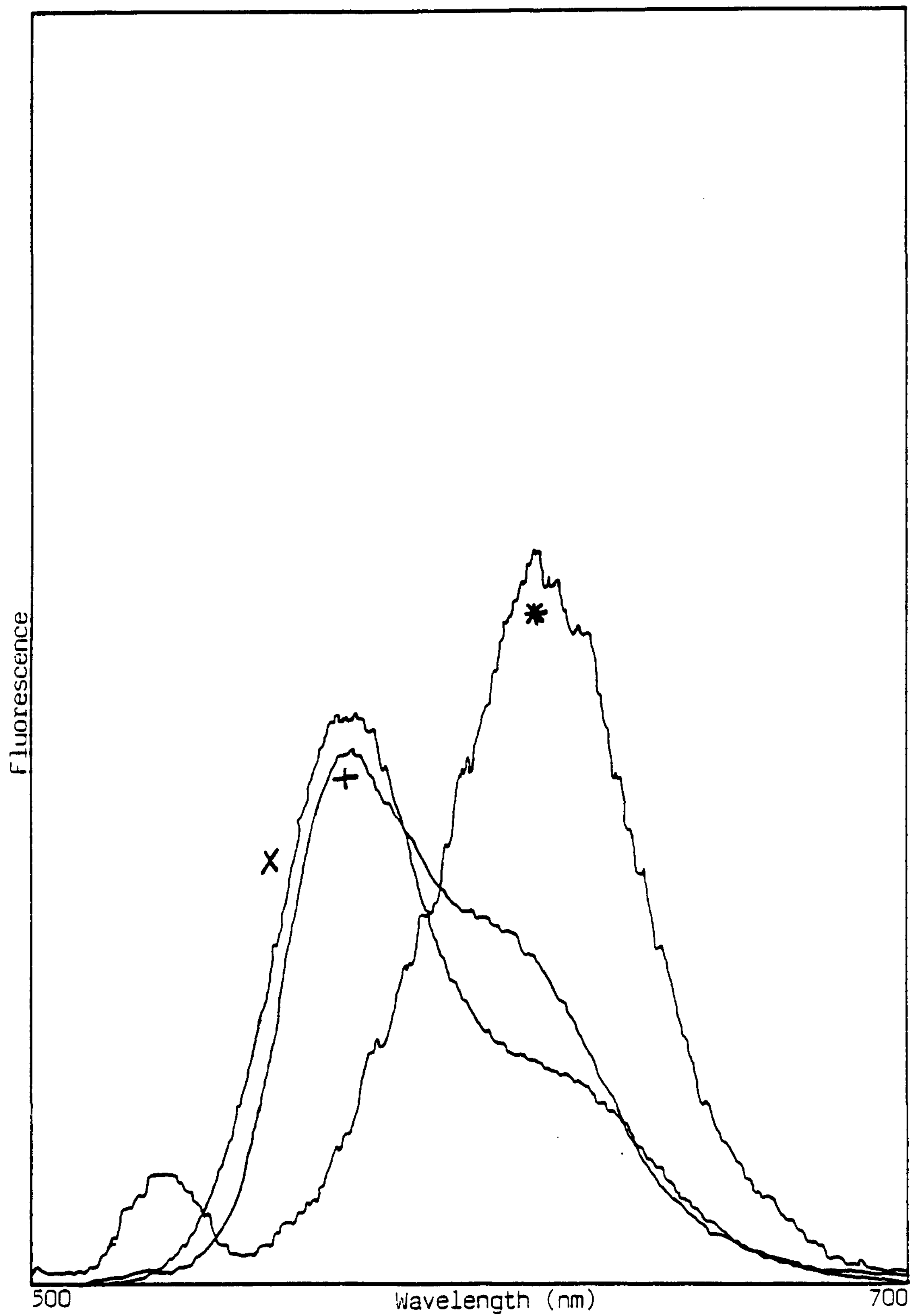


Figure 7. Right angle fluorescence spectra of rhodamine-6G.

FIGURE CAPTION

Figure 8. Right angle fluorescence spectra of rhodamine-6G. Solvent: 80% water, 20% methanol.

Concentration	Sensitivity Range
x - 5.2×10^{-6}M	0.3
+ - 5.2×10^{-5}M	1.0
* - 5.2×10^{-4}M	10.0

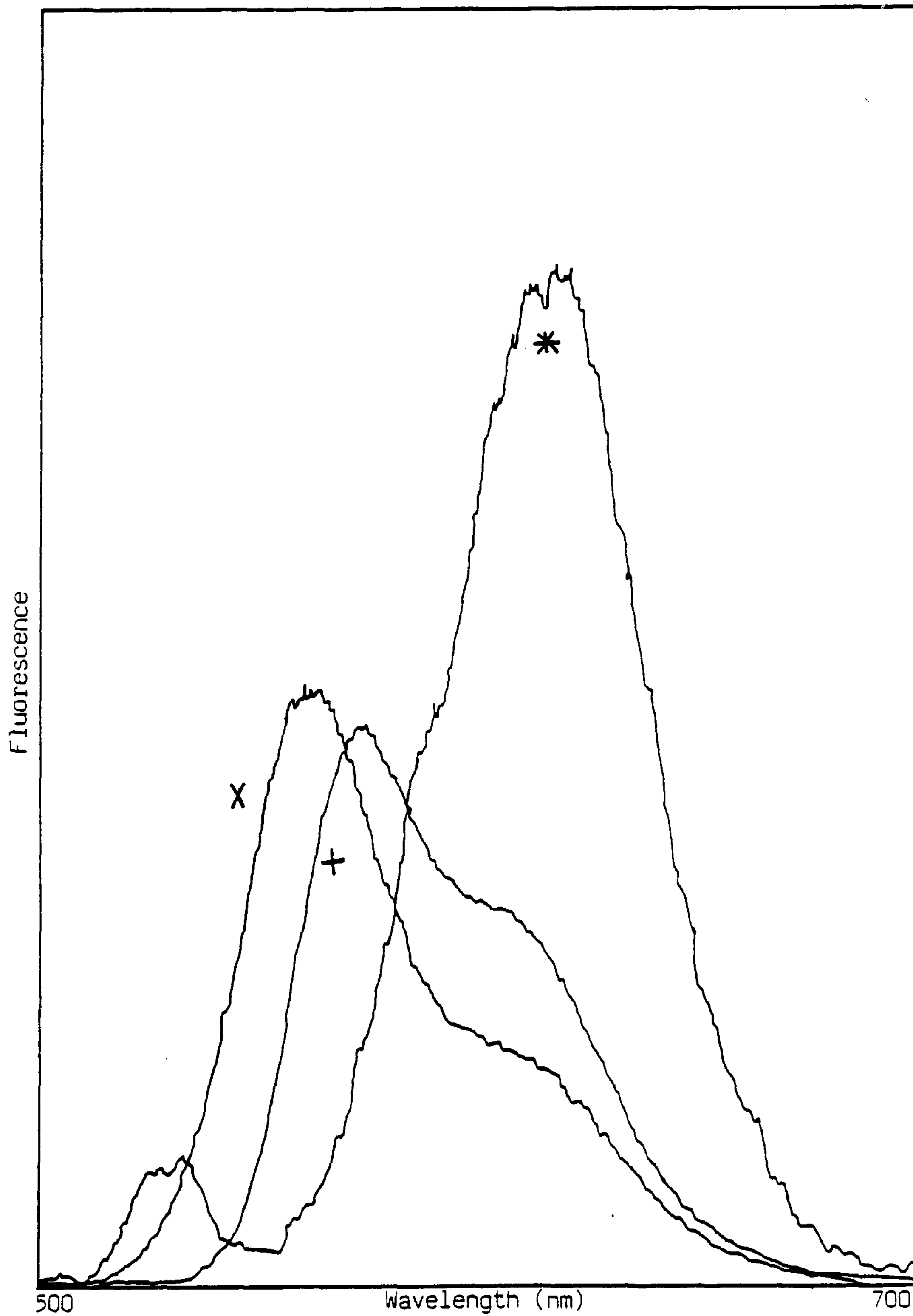


Figure 8. Right angle fluorescence spectra of rhodamine-6G.

FIGURE CAPTION

Figure 9. Right angle fluorescence spectra of rhodamine-6G. Solvent: 100% methanol.

Concentration	Sensitivity Range
x - 5.2×10^{-5}M	0.3
+ - 5.2×10^{-5}M	1.0
* - 5.2×10^{-4}M	3.0

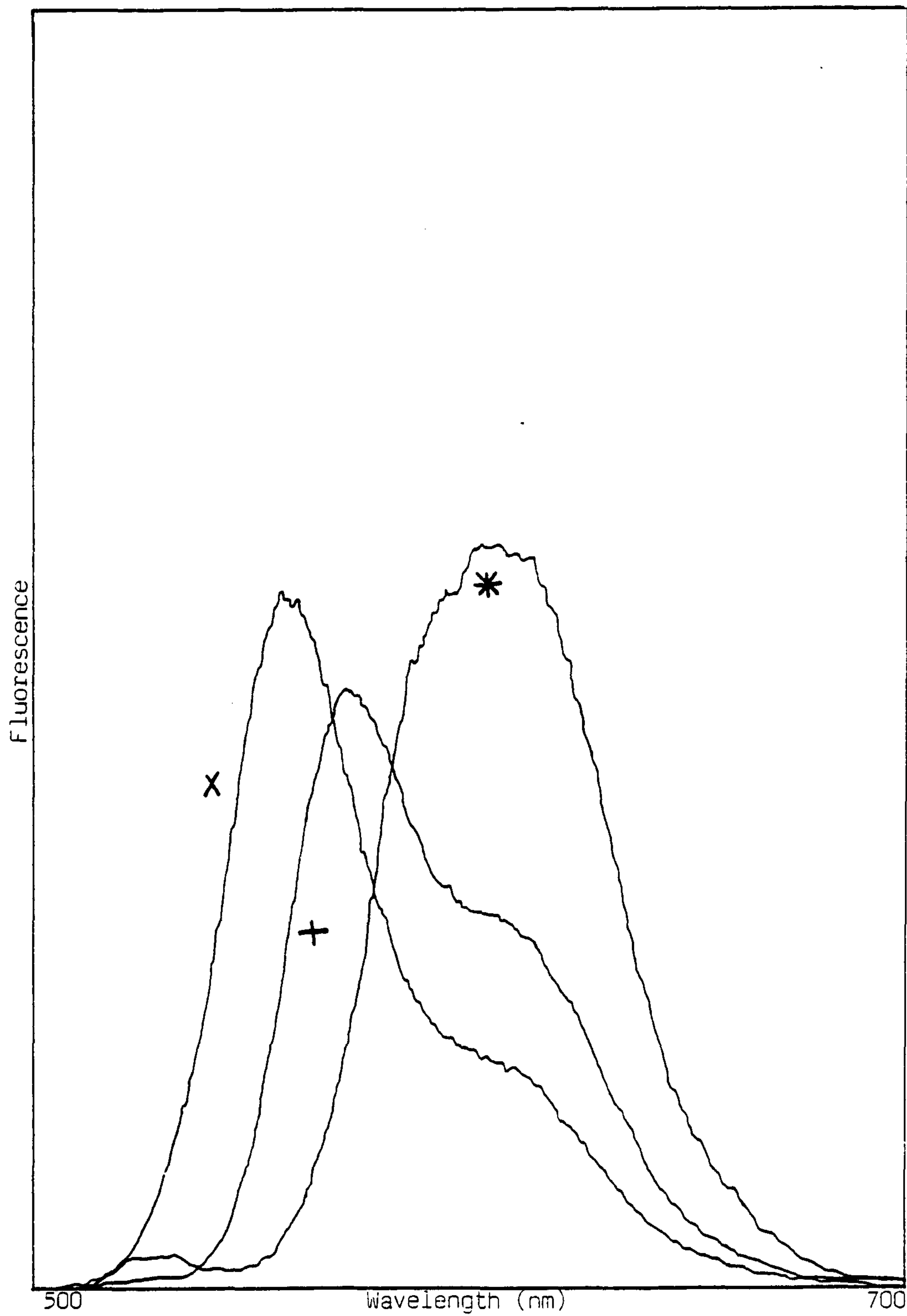


Figure 9. Right angle fluorescence spectra of rhodamine-6G.

FIGURE CAPTION

Figure 10. Dependence of fluorescence on concentration of rhodamine-6G. Solvent: 100% water, 90% water - 10% methanol, 80% water - 20% methanol, 100% methanol.

A - 100% water

B - 90% water, 10% methanol

C - 80% water, 20% methanol

D - 100% methanol

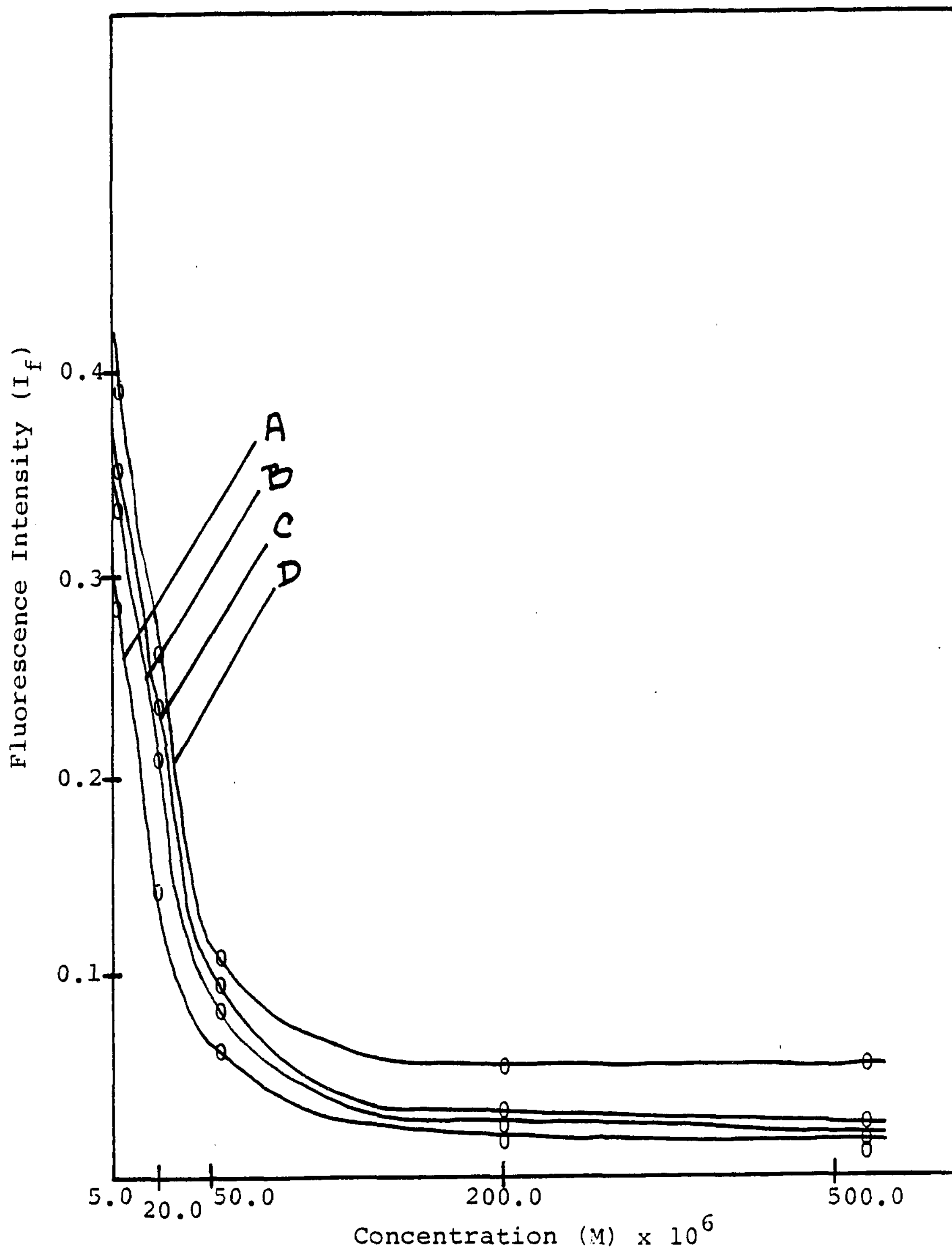
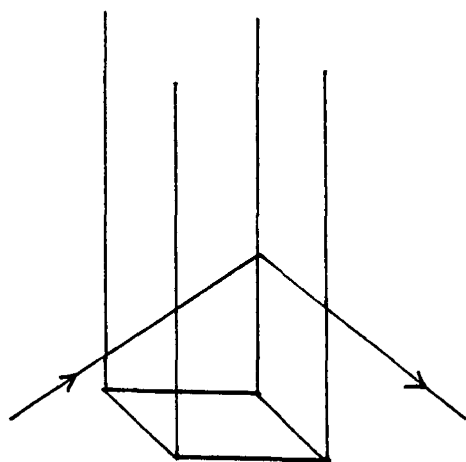


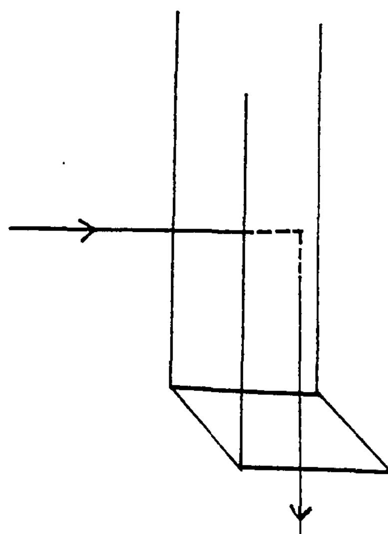
Figure 10. Dependence of fluorescence on concentration of rhodamine-6G.

the greater the fluorescence of solutions. Aggregation is favored in aqueous solutions; methanol promotes breaking of aggregates. Hence, a high methanol content dye solution is expected to have an increased monomer concentration, and thus, exhibit enhanced fluorescence. This suggests that from the chromatographic point of view, a high concentration of the dye and low methanol content of the mobile phase would be desirable.

Self-absorption and self-quenching influence fluorescence. Although there is some degree of self-quenching that increases with concentration, the observed changes of the fluorescence intensity of several orders of magnitude, with the concentration of the dye, are not primarily attributed to these effects. We believe that self-absorption is minimal in our systems for two reasons. Firstly, there is no significant absorption peak overlap at the emission wavelength range of the dye. Secondly, the results of front surface fluorescence studies we performed on our solutions are in general agreement with the right angle fluorescence data. (Figure 11).



A. Front Surface Fluorescence



B. Right Angle Fluorescence

FIGURE CAPTION

Figure 11. Front surface fluorescence spectra of rhodamine-6G. Solvent: 100% water.

Concentration: 5.2×10^{-6}M, 5.2×10^{-5}M, 5.2×10^{-4}M.

x - 5.2×10^{-6}M

+ - 5.2×10^{-5}M

* - 5.2×10^{-4}M

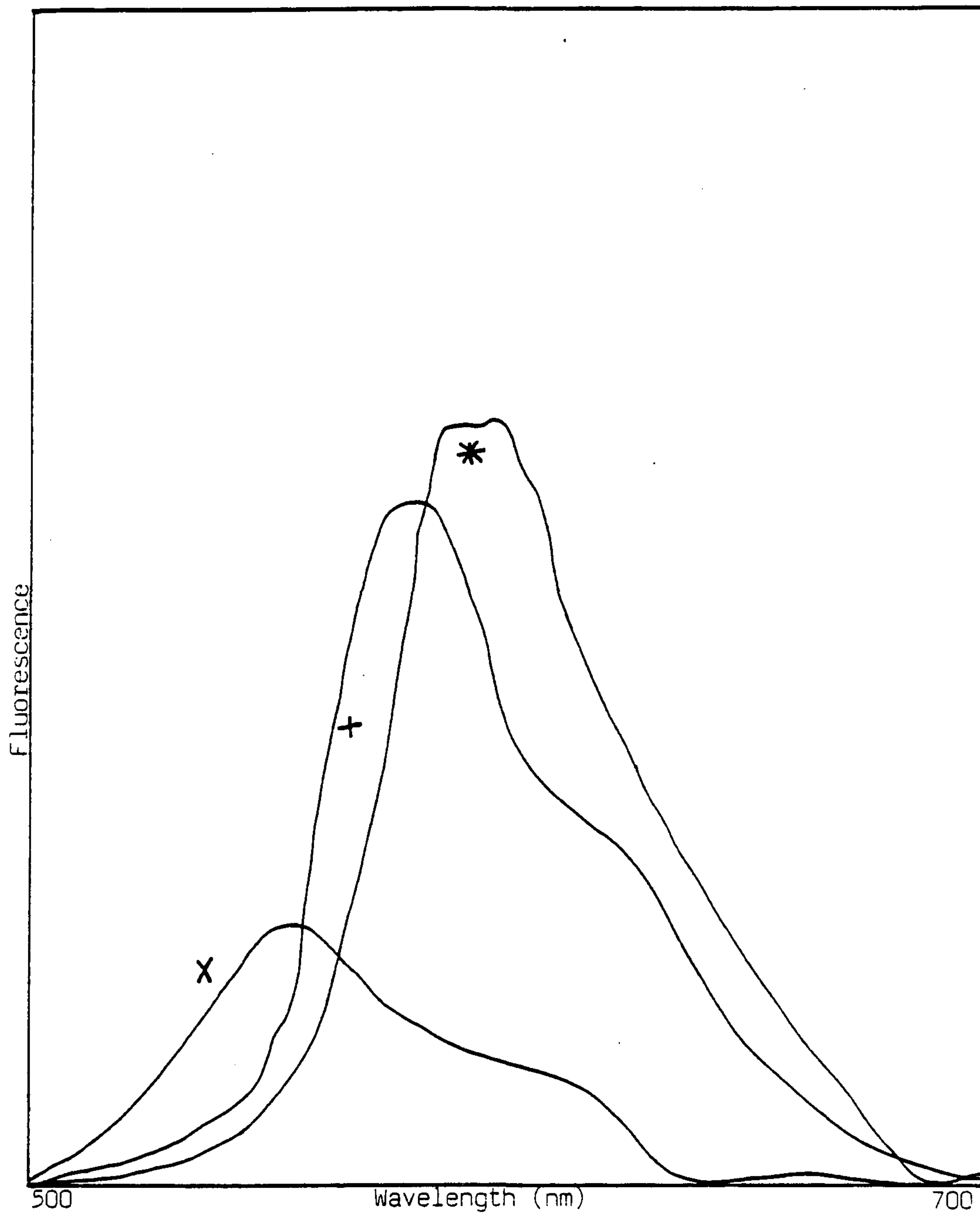


Figure 11. Front surface fluorescence spectra of rhodamine-6G.

Front surface fluorescence spectroscopy allows examination of the light emitted from the surface of the sample exposed. In right angle excitations, emitted radiation travels through the bulk of the sample before being detected. In this way, the technique of front surface illumination limits self-absorption effects.

Based on the data presented here, if we use a 5.2×10^{-4}M solution of rhodamine-6G as a mobile phase in chromatography, we would expect to detect an increase in fluorescence intensity due to separated analytes at 590nm.

CHAPTER 5

DETECTION OF ALIPHATIC ALCOHOLS IN REVERSE PHASE CHROMATOGRAPHY WITH THE AID OF RHODAMINE-6G

We have studied the manner in which the fluorescence of a rhodamine-6G solution is affected by the progressive addition of aliphatic alcohols. As the concentration of the alcohols in the dye solution increases, the fluorescence rapidly increases. When 1-Butenol is introduced to a 5.2×10^{-4}M solution of the dye at the one part per thousand concentration level, the fluorescence intensity of the latter is increased by 60%. No new bands are observed when alcohols are added to the dye.

We used the fluorescence of this dye to detect aliphatic alcohols separated by reverse phase chromatography. These analytes are well behaved both chromatographically and spectroscopically. The mobile phase of our system was 5.2×10^{-4}M of the dye solution in 90% water and 10% methanol. At parts per thousand concentration level the separated alcohols were detected at 590nm. Due to time and equipment limitations, higher concentrations were not investigated, and detection was not attempted by absorption.

According to our data, we expect the separated alcohols to shift the monomer-dimer equilibrium of the dye toward higher monomer concentration, resulting in enhancement of the fluorescence of the solution. The preliminary chromatographic experiments we performed, however, reveal an unexpected phenomenon. The separation of the

alcohols results in a decrease of the fluorescence intensity of the dye. Two possible explanations exist for this observation. It may be due to quenching of the fluorescence of the dye or; alternatively, it may also be that the separated alcohols are present in large enough amounts to partially wash the dye off the saturated column and thus increase the concentration of the mobile phase. If the first is true, it should become evident when the fluorescence of the dye is studied after excitation at wavelengths other than the one corresponding to maximum monomer absorbance. If the second possibility is true, then study of solutes in the parts per million concentration level should not exhibit the same problem, since very small amounts of alcohols will be present in the mobile phase.

CHAPTER 6

CONCLUSION

Rhodamine-6G dimerizes in water, increasingly so, as the concentration increases. Presence of methanol in the solvent causes partial breakup of dimers. The result of this action is enhanced absorption and fluorescence of the dye. These conclusions led us to believe that if a concentrated dye solution were used as a mobile phase in reverse phase chromatographic separation of aliphatic alcohols, we should be able to detect the latter, since they cause monomer formation and thus enhance the fluorescence intensity of the solution. However, we found that the result of such a separation is decreased fluorescence. This answers positively the detection capability question for this technique posed earlier and raises a question concerning the nature of the decreased fluorescence. In order to identify the source of this problem, it is necessary to firstly study the fluorescence of the dye after excitation at different wavelengths along the absorption bands, and secondly, to investigate detection of solutes at parts per million concentration level. The latter is also important for determining the sensitivity capabilities of this detection technique.

This study also suggests some other interesting experiments from the points of view of both analytical and physical chemistry. The fluorescence of rhodamine-6G should be studied in the context of intercavity detection of solutes. Furthermore, a study of diverse solutes can determine the selectivity limits of the technique and offer

insight into the nature of hydrophobic interactions and variation of solvent structure with added solutes. Detection by absorption of solutes in a mobile phase with dye should be explored for rhodamine-6G and other strongly absorbing dyes.

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