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I am submitting herewith a thesis written by Paul B. Huff, Jr., entitled "Selective Laser Detection Modes in Liquid Chromatography." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

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SELECTIVE LASER DETECTION MODES IN LIQUID CHROMATOGRAPHY

A Thesis

Presented for the

Master of Science

Degree

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Paul B. Huff, Jr.

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ABSTRACT

A fluorometric detection technique for liquid chromatography is described in Part I of this thesis. Fluorescence is monitored from a highly excited molecular state following sequential resonant excitation. Detection limits in the low nanogram range and linearity over three decades of concentration are demonstrated for the separation of several derivatized amines. Possible improvements in sensitivity and selectivity are also discussed.

The unique properties of the laser and the dependence of fluorescence signals on photon density for techniques excited by two photons are shown to facilitate miniaturization of flow cells without loss in signal.

Part II of this thesis presents three different modes of laser fluorometric detection for thin-layer chromatography. Detection limits in the low to mid picogram range and linearity over approximately three decades of concentration are demonstrated for the separation of several derivatized amines and oxadiazole compounds. The sensitivity and selectivity of these techniques along with the unique properties of the laser should result in future applications of laser fluorimetry in thin-layer chromatography detection.

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CHAPTER I

INTRODUCTION

Increasing concern about the carcinogenicity and toxicity of various nonvolatile organic compounds has led to a need for sensitive and selective detectors for liquid chromatography (1, 2). In the case of organic compounds exhibiting natural fluorescence, or those which can be made to fluoresce by derivatization, fluorometric detection is the most sensitive and generally selective liquid chromatography detector. Sensitivity for fluorescence detection is approximately 3 orders of magnitude higher than UV-visible absorbance detection (3). This increased sensitivity is due to the inherently low optical background associated with fluorometry. Background signals consist of contributions from Raman scattering, Rayleigh scattering, specular scattering, and eluent impurity fluorescence. Since conventional fluorescence from a compound occurs at longer wavelengths than that of excitation, it is possible to discriminate to a certain extent between the fluorescence signal and the background (4).

An important advantage of fluorometric detection is its selectivity. This selectivity can be attributed to the fact that while few compounds fluoresce at detectable levels, those that do, absorb and fluoresce at specific wavelengths providing spectra which can be used to identify various compounds.

Another advantage associated with fluorometric detection in liquid chromatography is its insensitivity to the composition and

flowrate of the eluent. This tends to lessen the effects on chromatographic baseline of gradient elution and pump noise which are significant problems in absorbance detection.

Laser Fluorescence Detection

Increased sensitivity and selectivity are often realized when a laser is used as an excitation source in fluorometric detection. Lasers with their characteristic spectral properties offer significant advantages over incoherent sources (5). Replacement of an incoherent excitation source by a laser in a conventional fluorometric detector does not automatically lead to improvements in detectability. Changes in the optical arrangement of the detector's flow cell will be needed to utilize the laser's spectral properties to their full extent. It therefore seems appropriate to expound in further detail on these properties in order to gain a better understanding as to what changes will be needed.

The property most often associated with lasers is their high output powers. Many commercially available lasers are capable of average powers of approximately 10 W and peak powers in excess of 1 MW. This is a significant improvements over the output of conventional light sources with equal bandwidths. Increasing the photon flux incident on a compound will increase the fluorescence signal up to the point of excited state saturation. This increase in fluorescence signal will not necessarily improve detectability, since a concomitant increase in the optical background will also occur. Thermal problems

associated with the nonradiative dissipation of absorbed excitation energy can also limit detection. Increases in optical background and thermal problems can be minimized by utilizing the spatial and temporal properties of the laser. Thus, a significant improvement in the sensitivity of fluorometric detection will result from the employment of laser excitation.

The properties of the collimated laser beam allow for the output of the laser to be focused to extremely small volumes. This has potential utility in fluorometric detection for "microscale" liquid chromatography, where ultralow volume detector flow cells are required. Another advantage of the collimated laser beam results from the fact that it is far easier to manipulate than the output from a conventional source and this can lead to a reduction in specular scatter.

Lasers are also unique in their ability to produce intense photon fluxes in short pulses. These pulsed lasers, when coupled with synchronous detection can be used to distinguish fluorescence signal from the Ramen and Rayleigh scatter of the eluent (6). Raman and Rayleigh scatter is coincident with excitation, while fluorescence has a finite lifetime after excitation. Rejection of Raman and Rayleigh scatter can be accomplished by adjusting the detector's response time to collect only that signal appearing after excitation. With the rejection of Raman and Rayleigh scatter, a decrease in background signal will result, leading to improved sensitivity.

The last property of the laser to be mentioned is the monochromaticity of the output. The highly monochromatic output of the

laser results in narrow Raman and Rayleigh bandwidths making it easier to spectrally reject those components of stray radiation. Conventional excitation sources may also be limited to similarly narrow bandwidths, but not without a severe reduction in output intensity.

Two-Photon Excited Fluorescence

The high output power of lasers makes it possible to observe a number of "nonlinear" spectroscopic processes which can be applied to liquid chromatography detection. One example of these "nonlinear" processes is two-photon excited fluorescence (TPEF). Two-photon excitation involves a resonant transition in a molecule, accomplished by the simultaneous absorption of two photons (7). One aspect of two-photon excitation is that it involves different selection rules than conventional one-photon excitation (8). Quantum theory states that for a transition to occur between two states, the transition moment integral must be nonzero. As a result, transitions between states of equal parity in centrosymmetric molecules are forbidden. This is not the case for two-photon absorption in centrosymmetric molecules where transitions between states of opposite parity are forbidden. The difference in the selection rules for one-photon and two-photon excitation can result in different excitation spectra for the two processes. This has the effect of providing an added dimension of spectral selectivity in fluorometric detection. An additional improvement in selectivity

arises from the fact that two-photon excited fluorescence involves three states, while conventional one-photon excited fluorescence requires only two. Since transitions between states of equal parity are forbidden, the direct radiative relaxation from the two-photon excited state to the ground state will not occur. An intermediate state of unsymmetric character is needed to satisfy the aforementioned parity selection rule. Therefore, relaxation from the two-photon excited states will first involve internal conversion to an unsymmetric state. This is illustrated in Figure 1.

The two-photon excitation process is a very inefficient process, and only through the use of high-powered lasers does it become analytically useful. As a result, this technique has been applied to liquid chromatography and been shown to provide satisfactory detection limits (9). The low detection limits obtained with this technique are the result of the low background levels associated with the two-photon excited process. This results from the fact that two-photon excited fluorescence occurs at wavelengths much shorter than those used for excitation, and this enables one to spectrally reject stray radiation that contributes to the optical background.

Sequentially Excited Fluorescence

The advantages in selectivity and detectability demonstrated by two-photon excited fluorescence are also exhibited by sequentially excited fluorescence (SEF). The SEF process is shown in comparison

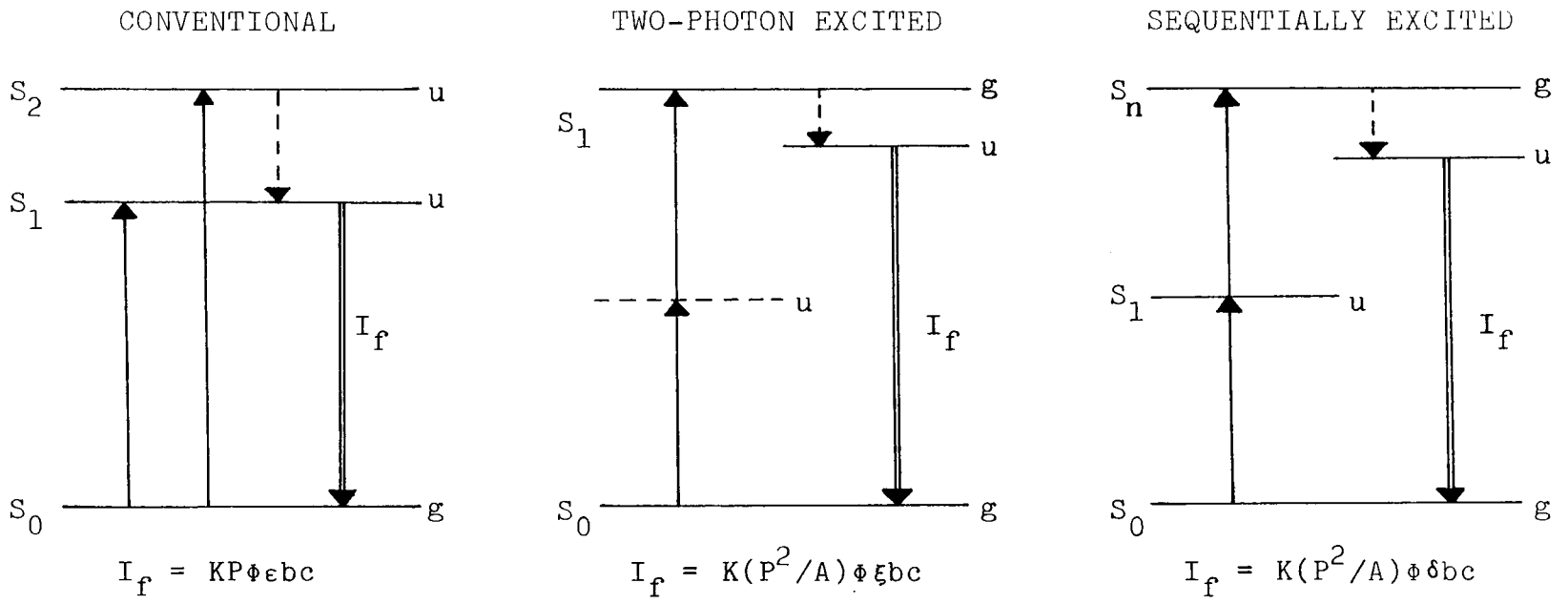

Figure 1. Electronic energy level diagrams of conventional fluorescence, TPEF and SEF.

to TPEF and conventional fluorescence in Figure 1. In the SEF process, a symmetric singlet ground state of a molecule is coupled, by two resonant absorptions, to a highly excited singlet state of the same symmetry. This sequential absorption of two photons can be shown as $S_o + h\nu_1 \rightarrow S_1^* + h\nu_2 \rightarrow S_n^*$, where S_o, S_1^* and S_n^* are the ground, first excited singlet, and some higher excited singlet states of a molecule, respectively. The resulting fluorescence is monitored from a highly excited singlet state of the molecule, as opposed to the S_1^* state as in TPEF or conventional fluorescence. As previously stated, direct emission from a symmetric excited state to a symmetric ground state is forbidden. Therefore, as in TPEF, internal conversion to an unsymmetric intermediate state is needed. The intermediate state can actually be S_n^*, since it can acquire the necessary symmetry through vibronic coupling with other states.

SEF is similar to TPEF in that it involves a highly inefficient optical process. Because of the proximity of electronic states between S_1^* and S_n^* and vibronic coupling of these states, internal conversion to S_1^*, and not fluorescence, is by far the most efficient means of depopulating S_n^*. This is in accordance with Kasha's rule, which states that "the emitting electronic level of a given multiplicity is the lowest excited level of that multiplicity" (19). The quantum efficiencies of highly allowed transitions from highly excited states can be estimated using rate constants for the various electronic transitions. The rate constant for internal conversion has an upper limit

equal to the molecular vibrational frequencies which are usually around 10^{13} S^{-1}. Radiative rate constants for highly allowed transitions are in the 10^{8} to 10^{9} S^{-1} range. Hence, the fluorescence quantum efficiencies for highly allowed transitions should have a lower limit of about 10^{-4} to 10^{-5} (10). Nevertheless, the low backgrounds inherent in SEF have permitted detection of the fluorescence from the highly excited states (11).

The intensity of the SEF signals can be estimated using kinetic equations which relate the number of SEF photons to the various experimental parameters. Included in these experimental parameters are the electronic rate constants for fluorescence (k_f), intersystem crossing (k_x), and internal conversion (k_{ic}). The rate constant for the depopulation of the electronic state S_n^* can be expressed as

$$k_{d_n} = k_{f_n} + k_{x_n} + k_{ic_n} \qquad (1)$$

If a single continuous wave laser is used for both the S_o to S_1^* and S_1^* to S_n^* transitions, then steady state rate equations can be solved to yield

$$I_{f_n} = K \frac{P^2}{A} \frac{k_{f_n}}{k_{d_1} k_{d_n}} \delta b C \qquad (2)$$

where I_{f_n} is the SEF intensity, K is an instrumental efficiency factor, P is the laser output power, A is the cross-sectional area of the focused laser beam, δ is a sequential absorption coefficient, b is

the path length, and C is the concentration (10). This equation only holds true when the excited states are not saturated. The equation must be modified if a pulsed laser source is used for excitation. For a laser with large pulse widths this modification consists of multiplying the equation by the duty cycle of the laser. In the case of excitation pulse widths that are much shorter than the first excited state lifetime, I_{f_n} will be dependent on excitation pulse energy instead of excitation pulse power, and independent of k_{d_1}.

CHAPTER II

EXPERIMENTAL SECTION

Sample Preparation

Several alkyl and aromatic amines were derivatized with 2-chloro-4-nitrobenzo-2 oxa-1,3-diazole (NBD-Cl) prior to separation. NBD-Cl is a fluorogenic reagent that reacts with amines to form highly fluorescent 7-substituted derivatives (12). Approximately 1.0 μℓ of each amine was added to a solution containing 5.0 ml of 10 mg/ml NBD-Cl in methanol, 5.0 ml of methanol saturated with NaOAc, and 5.0 ml of deionized water with a pH of 9. The resultant mixture was placed in a closed container and heated to 55°C for a period of one hour. Successive dilutions of these stock solutions were made with HPLC grade methanol. Stock solutions of various oxadiazole compounds were made by dissolving a weighed amount of the compound in HPLC grade methanol.

Chromatography

A Rheodyne 20 μℓ fixed loop injector was used to inject the derivatized amines onto an Alltech 10 μm, 4.6 mm x 25 cm C_{18} chromatographic column. The eluent was delivered at 1.0 ml/min by an Altex reciprocating piston pump. The composition of the eluent was changed from 60% to 75% HPLC grade methanol in distilled water at 10 minutes into the separation. The chromatogram was recorded using a strip chart recorder.

Detection

Fluorescence from derivatized amines was detected using the arrangement shown in Figure 2. Excitation was provided by either a Spectra Physics argon ion laser operating at 488 nm or a National Research Group N_2-pumped dye laser tuned to 488 nm using Coumarin 481 dye. The argon ion laser was operated at 1-3 W. The N_2-pumped dye laser was operated at 60 Hz with an average power of 10 mW and peak power of approximately 35 kW. The laser radiation was first passed through a Corning GG-455 sharp cutoff filter. This was done in order to block laser plasma lines from the expected region of blue shifted emission. The excitation radiation was focused with a 25 mm focal length quartz lens into a 1.0 mm I.D. x 2.0 mm O.D. Suprasil quartz capillary flow cell. The flow cell was positioned by a Newport Research Corp. fiber optic positioner. The SEF emission was collected and collimated at a 90° angle from the incident excitation radiation with a f/1, 25 mm focal length quartz lens. The collimated SEF emission was passed through and isolated by three Corning 7-54 bandpass filters and a 2.0 cm saturated $CuSO_4$ cell. The spectral transmittance characteristics of these filters are illustrated in Figure 3.

The isolated SEF emission was detected with a RCA 1P28 photomultiplier tube (PMT) operated at 900 V. The resulting current produced by the PMT was measured by a Pacific Precision Instruments quantum photometer operated in either a photon counting or nanoampere

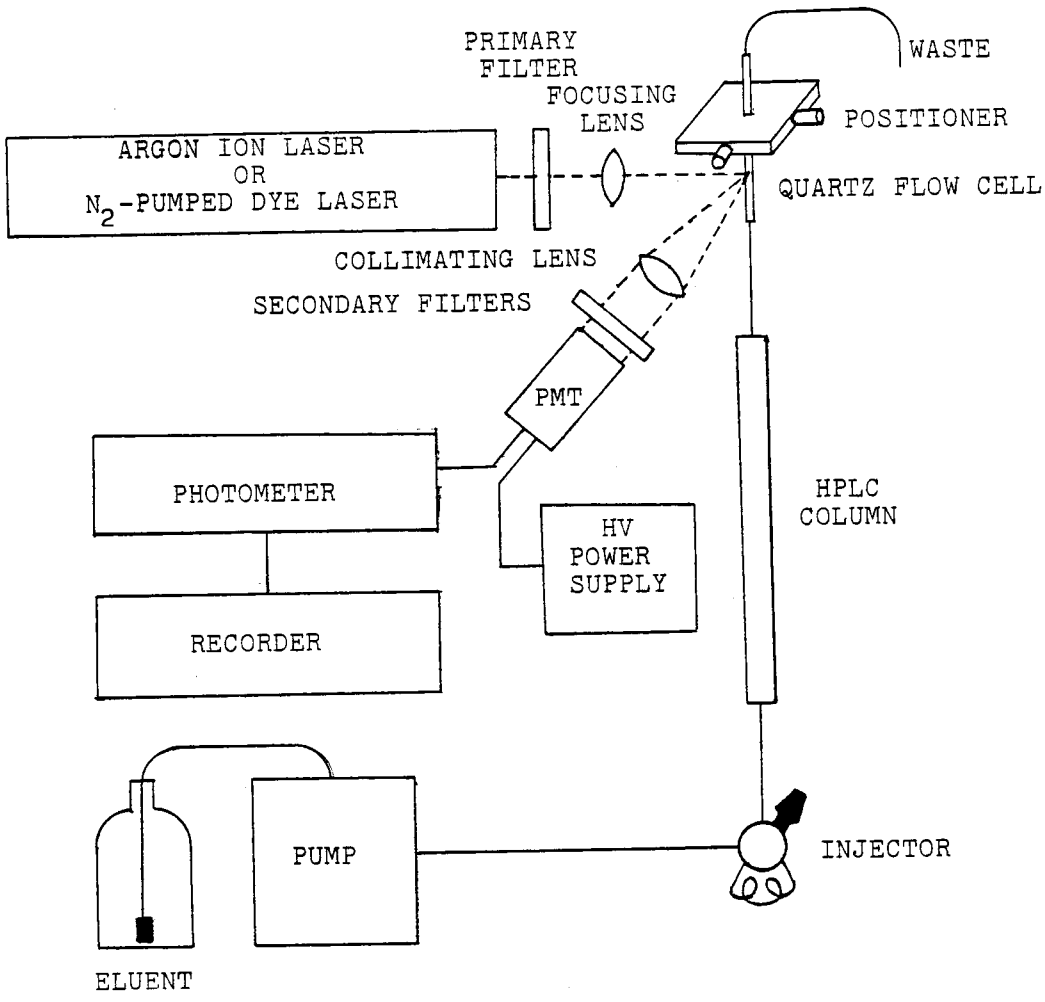

Figure 2. Apparatus used for SEF detection in HPLC.

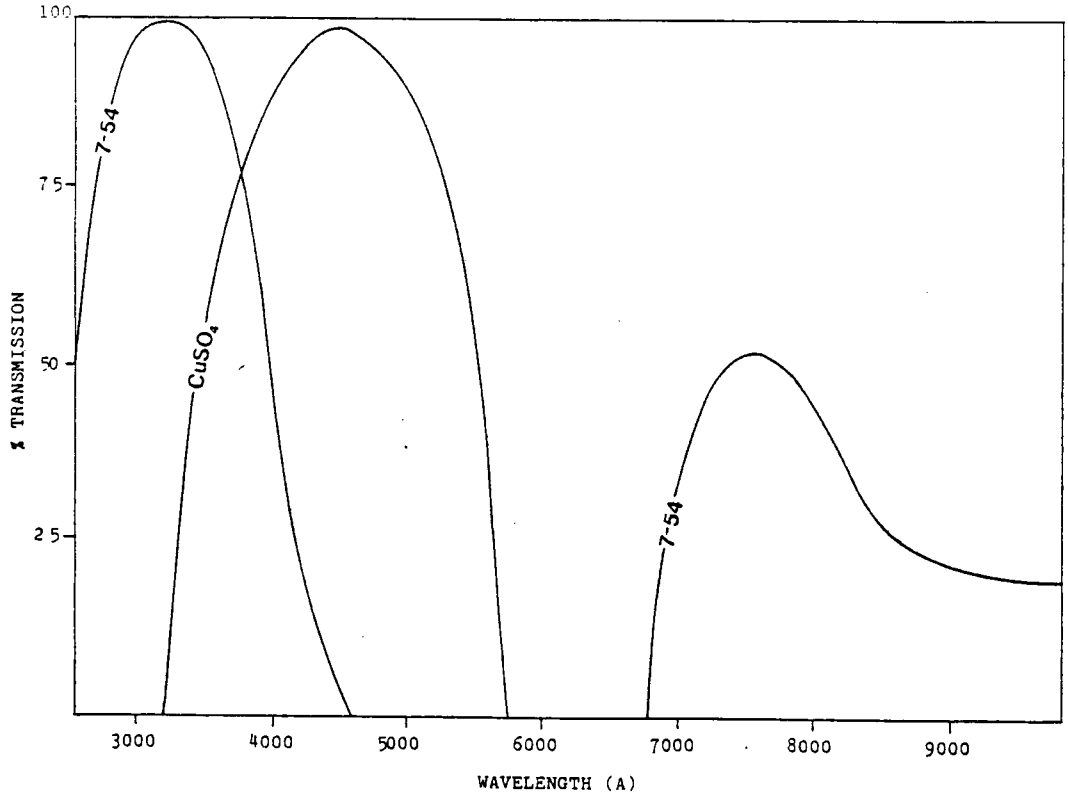

Figure 3. Spectral transmittance characteristics of emission filters.

measuring mode. The photon counting mode was used with the argon ion laser as the excitation source. The nanoampere measuring mode was used with the N_2-pumped dye laser since the quantum photometer could not resolve the large number of photon-induced PMT current pulses, coincident with the laser pulse, in the photon counting mode. However, even in the nanoampere measuring mode, the pulsed excitation source results in signals that were partially shunted to ground by a protective diode clamp at the photometer input. In order to decrease this effect, the feedback capacitance for the current measuring operational amplifier of the electrometer was increased. The change in the chromatographic peak shapes produced by the slowed photometer response was negligible.

A flow cell miniaturization study was performed using the previously described arrangement which included the argon ion laser as the excitation source. The laser was operated at 1.0 W using the 488 nm line with a half angle divergence of 0.56 mrad. The argon ion was preferred over the N_2-pumped dye laser because of its higher quality beam. The laser radiation was focused with a variety of glass or quartz double convex and plano-convex lenses ranging in focal length from 18 mm to 200 mm. The laser radiation was focused with each of these lenses into one of three Suprasil quartz capillary flow cells (1.0 mm I.D. x 2.0 mm O.D., .50 mm I.D. x 1.0 mm O.D., or .25 mm I.D. x .50 mm O.D.). The optimum signal obtained for each lens flow cell combination was recorded.

In order to optimize the ratio of SEF signal to background noise level, a 10^{-3} M solution of the scintillator 2-(4-biphenyl)-5-phenyl-1,3,4-oxadiazole (PBD) was used. PBD is a two-photon absorber that provides a strong fluorescent signal (blue-shifted emission) with minimal thermal distortion. The optical components were aligned and focused while the PBD solution was pumped through the quartz capillary flow cell. The alignment and focusing of the optical components is critical in SEF detection. This is mainly due to the power density dependence of the SEF process. The focused laser beam must therefore pass through the center of the flow cell thus maximizing power and reducing specular scatter. Positioning of the beam within the flow cell was accomplished using a Newport Research Corp. translation stage. Although not as critical as the focusing lens, the collimation lens must also be positioned by a translation stage. This is done in such a way as to collect and collimate the largest possible signal onto the PMT. Once optimized, the previously described setup was found to be stable, resulting in reproducible signals.

Injections of 20 μℓ amounts of a stock mixture of derivatized amines were made onto the column using the previously described chromatographic parameters.

CHAPTER III

RESULTS AND DISCUSSION

Sensitivity

The detection limit of the cyclohexylamine derivative, based on a $\frac{S}{N}$ of 3, was 0.75 ng. This is about three orders of magnitude poorer than that obtained by conventional fluorescence. Although the detection limits for all derivatized amines were not determined, they were extimated to be in the low nanogram range.

The observed background levels of SEF detection were near the dark current of the PMT. These low background levels can be attributed to the large blue shift between excitation and emission wavelengths which allows one to spectrally reject stray radiation contributions. The rejection of these stray radiation contributions is more difficult for the case of conventional fluorescence. As a result, increases in excitation intensity which can lead to larger conventional fluorescence signals can often be limited by concomitant increases in stray radiation. As shown in equation 2, SEF has an additional advantage of having a quadratic dependence on excitation intensity. This is demonstrated in Figure 4 for the separation of several derivatized amines operating the argon ion laser at 1 W and 3 W. As a result, improvements in detectability resulting from an increase in excitation intensity, are more likely to occur with SEF detection. However, at some point stray radiation will be produced which cannot be spectrally

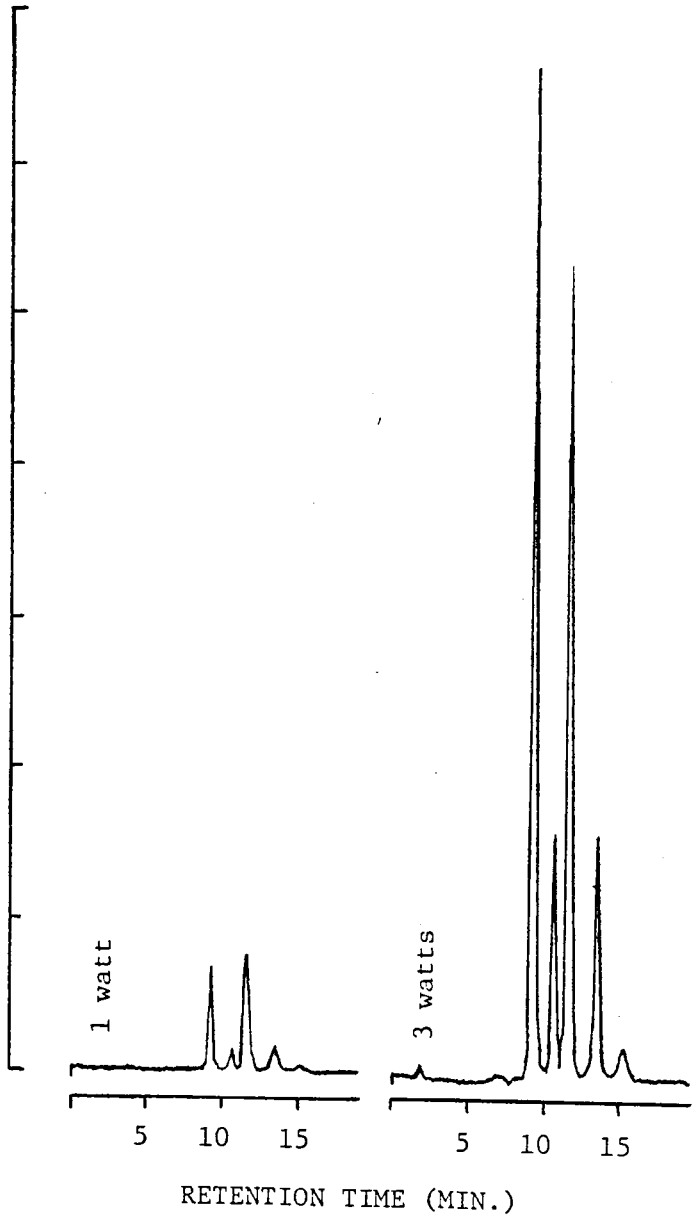

Figure 4. Laser power dependence of SEF signal.

rejected, and even SEF detection will become background limited. One must also consider the possibility of photolysis occurring when increasing the excitation intensity. This will ultimately place a limit on the excitation intensity which can be used.

Both a continuous wave laser and pulsed laser were used for excitation in SEF detection. Sample chromatograms utilizing both excitation sources are shown in Figure 5 for the separation of several NBD derivatized amines. The peak height to noise ratio for the pulsed excitation case and the continuous wave excitation case are similar, even though the average power of N_2-pumped dye laser is 200 times less than that of the argon ion laser. Significant improvements in SEF signal should therefore arise from the employment of more powerful pulsed lasers, such as excimer-pumped or Nd:YAG-pumped dye lasers. Additional improvements in detectability could also occur with a decrease in background levels. For SEF detection, this can be accomplished by cooling the PMT and gating the detector.

Linearity

The linear dynamic range of conventional fluorometric analysis is usually limited by preabsorption and postabsorption. These two undesirable effects are minimized by the low volume flow cells used in liquid chromatography. In SEF detection the linear dynamic range is limited by thermal distortion of the laser beam, which results from a large amount of absorbed excitation radiation being nonradiatively dissipated. This nonradiative decay generates a localized temperature

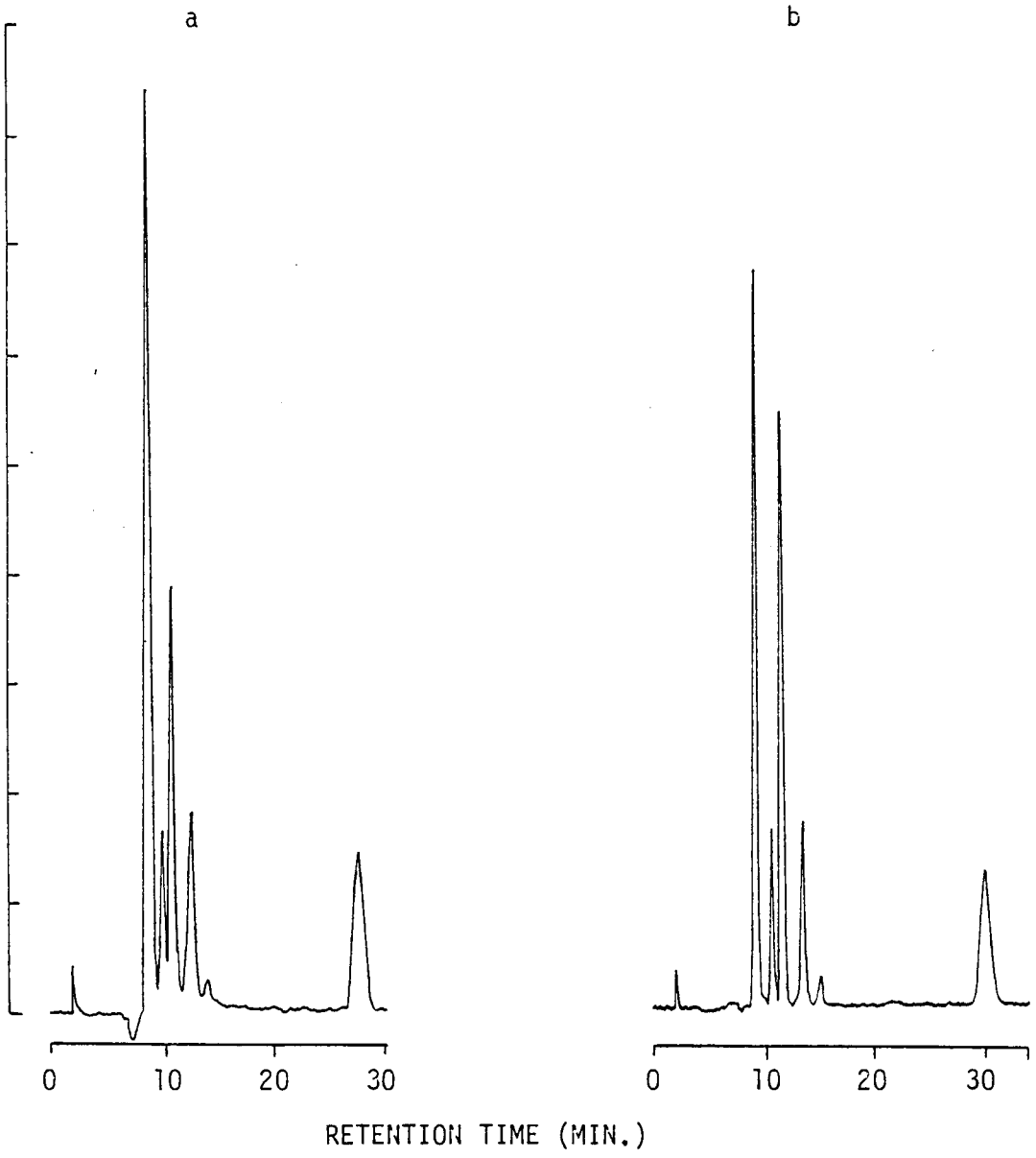

Figure 5. Comparison of pulsed laser excitation (a) and continuous wave laser excitation (b) for the SEF detection of several derivatized amines.

increase which changes the refractive index of that area. This area of distortion increases the cross-sectional area of the focused laser beam, resulting in a decrease in SEF signal as predicted in equation 2.

The thermal effects can be mitigated by employing pulsed lasers with low average powers. This was visually confirmed by observing the distortion of the laser beam as a concentrated solute band passed through the flow cell. The observed beam distortion of the N_2-pumped dye laser was less than that observed for the argon ion laser. While pulsed laser excitation reduces thermal effects, it has the disadvantage of producing the aforementioned problem of detector saturation. Figure 6 shows calibration plots for SEF detection of NBD derivatized cyclohexylamine utilizing both a continuous wave laser and pulsed laser for excitation. Correlation coefficients of 0.9812 and 0.9957 were obtained for the case of excitation with the argon ion laser and the N_2-pumped dye laser, respectively. Detector saturation occurring with pulsed excitation and thermal problems resulting from continuous wave excitation (i.e., high average power) limit the linear dynamic range of these detectors. A drift in laser power was noticed for the case of excitation with the N_2-pumped dye laser. Normalization with a reference standard should negate this problem.

Selectivity

Several factors influence the chromatograms obtained when using SEF detection. The differences observed between SEF chromatograms and conventional fluorescence chromatograms can mainly be attributed to

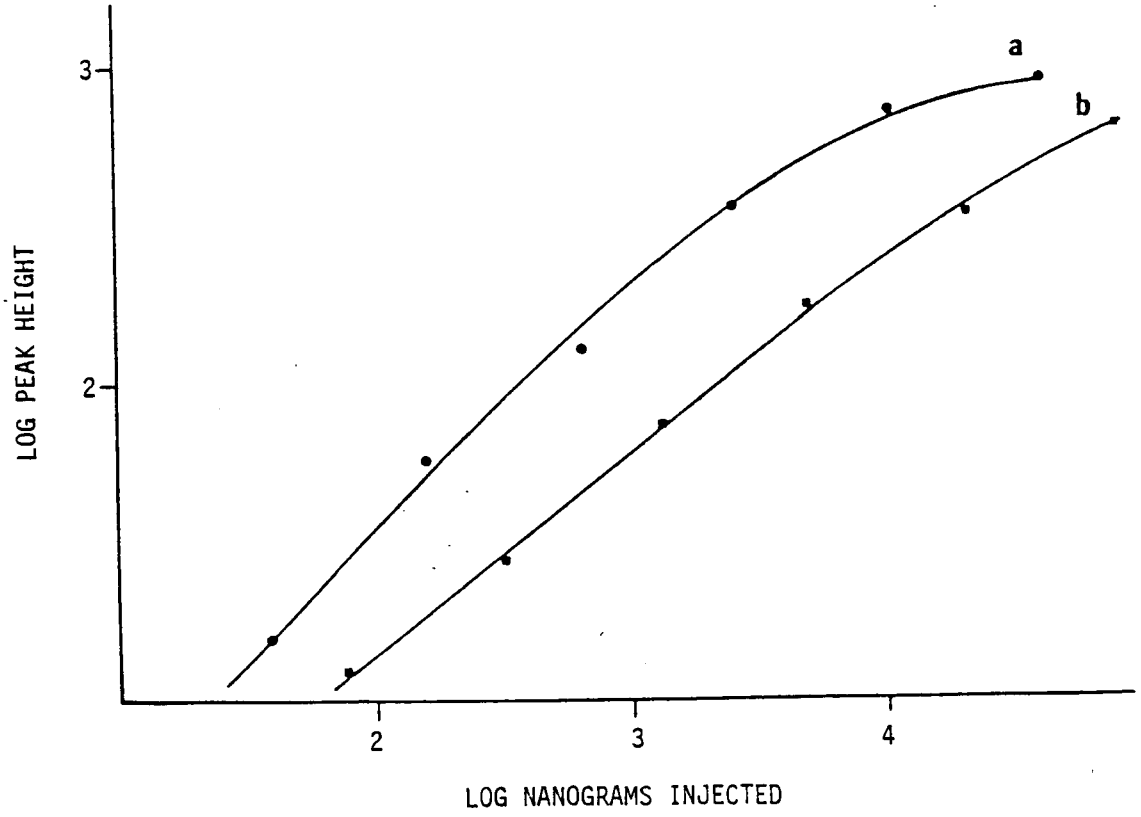

Figure 6. Calibration plots for NBD-cyclohexylamine using argon ion laser excitation (a) and N_2-pumped dye laser excitation (b).

the differences in excited-state symmetry for the two processes. This has been demonstrated by Sepaniak and Yeung in the detection of coal liquids separated by high performance liquid chromatography (13). Other factors contributing to the difference are the absorption coefficients and excited-state lifetimes of the compounds of interest. A large S_0 to S_1^* absorption coefficient and a large fluorescence quantum efficiency are critical for a large conventional fluorescence signal. A large S_1^* population is also necessary for SEF. This, however, can be accomplished without a large S_0 to S_1^* absorption coefficient, by instead having a long S_1^* lifetime. From this, one can conclude that molecules with $n\pi^*$ lowest excited singlet states, which usually have low S_0 to S_1^* absorption coefficients and long S_1^* radiative lifetimes, could give large SEF signals.

Another aspect of the selectivity of SEF that should be mentioned is the polarization information present in the SEF signal. The short lifetime of the sequentially excited state results in the emission being essentially coincident with the S_1^* to S_n^* absorption. The absence of molecular motion during this short lifetime allows for the utilization of the polarization information present in the transition. This aspect of selectivity has been shown to provide distinctive sequential excitation polarization spectra for selected xanthene dye solutions (14). Thus, the prediction can be made that this type of polarization information could be used to aid in the identification of coeluting compound in liquid chromatography.

Flow Cell Miniaturization

Efforts to increase the efficiencies of chromatographic columns have led to the development of "microscale" columns (15). Efficiencies of 650,000 theoretical plates have been demonstrated for the separation of various alkylbenzenes using "microscale" columns (16). These columns have the potential for even greater efficiencies, but have been limited by the availability of ultralow volume detectors. The effects of band dispersion in detector cells have been well studied by various researchers (17). In some cases, a detector flow cell volume of less than 10 nL would be needed before the chromatographic efficiency is limited by the column and not the detector (18). Unfortunately, most optical detector signals depend upon the optical path length, which must be reduced in order to achieve these ultralow volumes. Even with the use of laser sources, this can result in prohibitive reductions in the optical signals.

Equation 2 states that the fluorescence signal generated by two-photon excitation (i.e., SEF and TPEF) depends inversely on the cross-sectional area of the laser beam in the flow cell. The cross-sectional area (A) of a focused Gaussian laser beam can be approximately given by

$$A \approx \pi f^2 \theta^2 \qquad (3)$$

where f is the focal length of the focusing lens and θ is the half-angle divergence of the laser beam. This focused cross-sectional area can be

maintained over a uniform distance called the Rayleigh range (Z). The Rayleigh range is given by

$$Z = A/\lambda \qquad (4)$$

where λ is the wavelength. The SEF and TPEF signals should increase with a decrease in the focal length of the focusing lens, until the Rayleigh range is equivalent to the optical path length of the flow cell. Any further reductions in the focusing lens focal length will only cause difficulties in passing the laser beam through the flow cell. Thus, reductions in the path length of the flow cell in SEF and TPEF detectors can be compensated for by simply choosing a shorter focal length lens.

A study of the effect of focal length and cell diameter on signal levels was made to determine the applicability of SEF and TPEF detectors to "microscale" columns. Excitation was provided by the argon ion laser. The decision to use the argon ion laser was based upon the superior beam quality and absence of detector saturation associated with continuous wave excitation. A dilute solution of PBD was used in order to eliminate problems with thermal distortion. While SEF and TPEF display the same dependence on laser power density, they differ in the amount of thermal distortion they produce.

The results obtained in this study are shown in Figure 7. The observed TPEF signal was found to increase with the inverse square of the focusing lens' focal length. The maximum signal obtained for each of the three flow cells was approximately the same. The slight

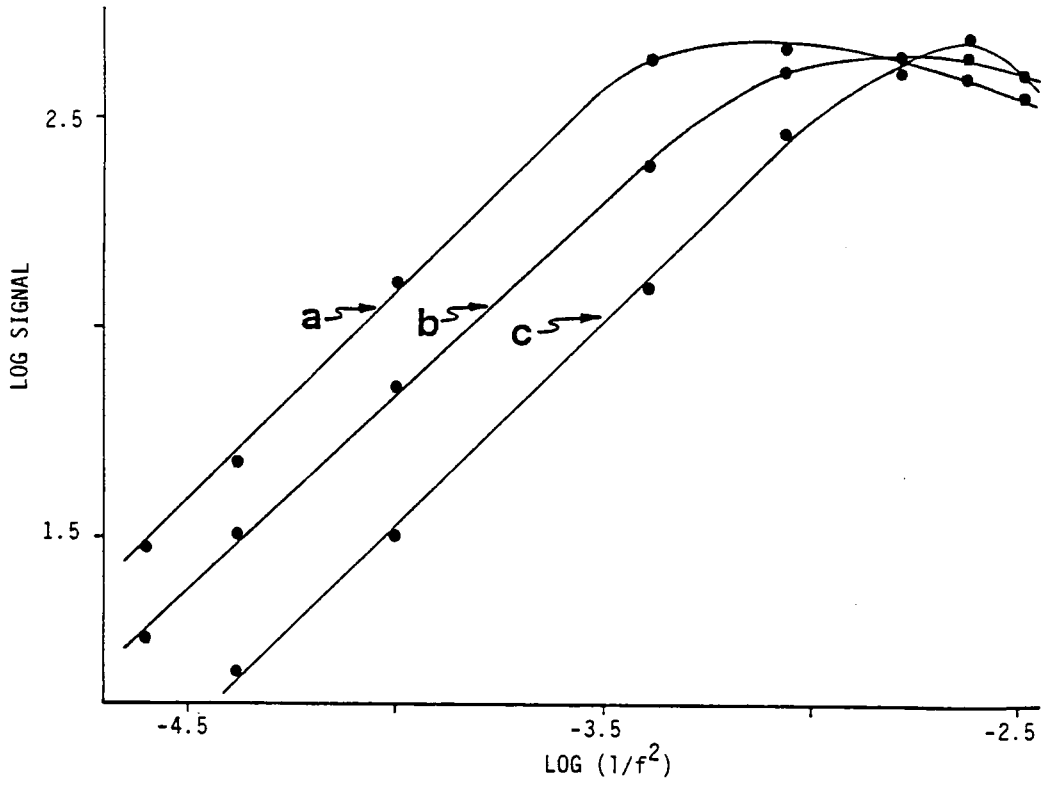

Figure 7. Logarithmic plot of the two-photon excited fluorescence signal of a dilute PBD solution vs. the inverse square of the focusing lens focal length (f). Flow cell dimensions are (a) 1.0 mm I.D. by 2.0 mm O.D., (b) 0.50 mm I.D. by 1.0 mm O.D., and (c) 0.25 mm I.D. by 0.50 mm O.D.

decrease in signal observed for the shorter focal length lenses was thought to be caused by alignment problems associated with focusing the laser beam through the flow cell.

Laser beam divergence and the availability of lenses can limit the size of flow cells used in SEF and TPEF detectors. Still, the prediction can be made that the sensitivity of SEF and TPEF detection should become more competitive with that of conventional fluorescence detection when used with "microscale" columns.

CHAPTER IV

CONCLUSIONS

A laser-based detection technique for liquid chromatography has been presented which probes different excited states than conventional fluorescence detectors. While SEF has demonstrated sensitivity poorer than conventional fluorescence, the unique selectivity of this technique has been previously demonstrated. The low sensitivity of SEF detection currently limits the value of this technique in real analysis. The use of state of the art pulsed lasers and signal recovery equipment should result in increased sensitivity, thus making this technique more competitive.

The real value of SEF detection will become evident in the area of "microscale" columns. The use of "microscale" columns for the separation of increasingly complex samples will eventually lead to decreases in column diameter and the need for ultralow volume detectors. As a result, one can predict that this technique will find its greatest use in the area of "microscale" liquid chromatography.

PART II

LASER FLUOROMETRIC MODES OF DETECTION IN THIN-LAYER CHROMATOGRAPHY

CHAPTER I

INTRODUCTION

Improvements in liquid chromatography columns and equipment over the past decade have greatly increased the separating power of that technique and have resulted in the use of the descriptive term high performance liquid chromatography (HPLC). Similarly, the term high performance thin-layer chromatography (HPTLC) has been adopted as a result of recent technological advances, which have increased the separating power of thin-layer chromatography (20). Despite the exceptional separating efficiencies attained with HPTLC, the fraction capacity of the technique is generally low relative to HPLC. However, the static nature of developed thin-layer plates facilitates the use of multidimensional detection, which can minimize the limitation of low fraction capacity. Thus research into new modes of selective detection is perhaps even more important in the development of HPTLC than it has been in HPLC.

Components separated by thin-layer chromatography are generally detected photometrically, using either absorbance or fluorometric modes of operation (21). Lasers have been employed in the fluorometric detection of aflatoxins separated by thin-layer chromatography (22, 23). Beam collimation and high power are unique spectral characteristics of the laser, which can be particularly beneficial for fluorometric detection in thin-layer chromatography. Separated component spot

diameters in HPTLC can be as small as one mm (24). It is not possible to focus conventional light sources to these dimensions without prohibitive losses in power, however, the collimated beams of lasers can be easily focused to beam diameters less than 100 μm. The high powers available with lasers can result in significant signal increases in fluorometric detection. Moreover, high laser power makes it possible to utilize certain nonlinear excited fluorescence processes in thin-layer chromatography detection.

Two-photon excited fluorescence and sequentially excited fluorescence are two nonlinear excited fluorescence techniques which have been described in Part I of this thesis. The feasibility of nonlinear excited fluorescence and conventional laser excited fluorescence as fluorometric detection modes for thin-layer chromatograms will be demonstrated. The advantages and limitations of each will also be discussed.

Apparatus

Separated components were detected using the apparatus shown in Figure 8. Excitation was provided by a N_2-pumped dye laser tuned to 488 nm using Coumarin 481 dye. The average output power of the laser was 8 mW (peak power approximately 25 kW) when operated at 60 Hz. The laser radiation was initially passed through a Corning GG-455 sharp cutoff filter, which removed interfering N_2 laser radiation from the emission region. The laser radiation was then focused onto

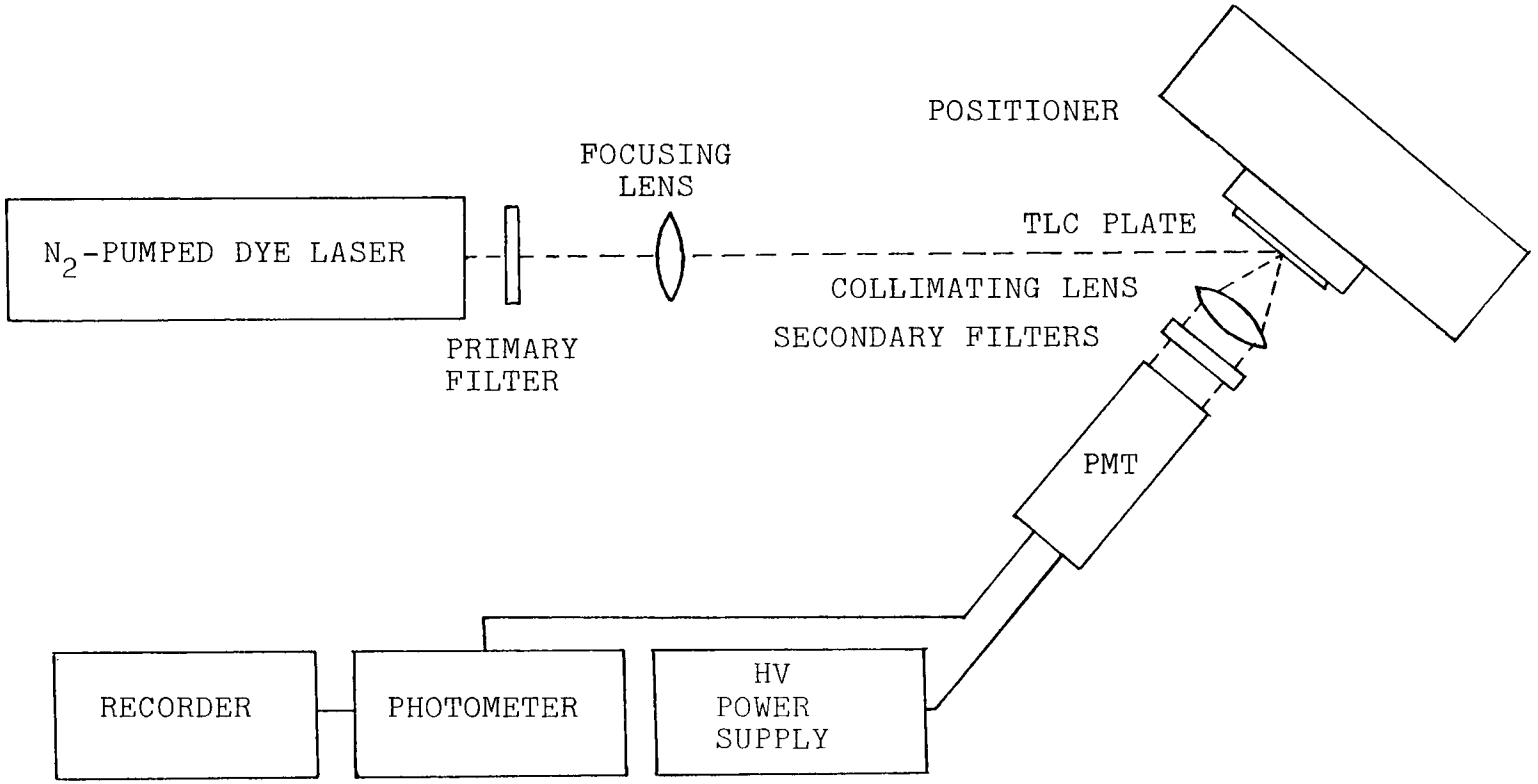

Figure 8. Schematic diagram of laser-based fluorometric detector for thin-layer chromatography.

the thin-layer plate at an angle of 45° using a 200 mm focal length lens. The thin-layer plate was scanned in front of the focused laser beam at approximately six mm/min using a modified syringe pump (Harvard Apparatus Co., Inc., Dover, MA, Model 600-900).

Fluorescence emission was collected at an angle of 45° from the incident excitation radiation, with a f/1, 25 mm focal length quartz lens. A combination of three Corning 7-54 bandpass filters and a two cm saturated $CuSO_4$ cell was used to isolate the TPEF and SEF emission. For the detection of the conventional fluorescence, the 7-54 bandpass filters and $CuSO_4$ cell were removed and the emission was passed through a Corion OG 570 sharp cutoff filter and a monochromator (Instruments SA, Inc., Metuchen, NJ, Model H 20). The monochromator was operated at 585 nm with a 6 nm bandpass.

The isolated emissions for the TPEF and SEF techniques were detected with a RCA 1P28 photomultiplier tube (PMT) operated at 850 V and mounted in a dry ice cooled housing. For the detection of the isolated conventional fluorescence emission, the PMT voltage was reduced to 650 V and the PMT was not cooled. The photocurrents were measured with a quantum photometer operated in a nanoampere measuring mode. A 10 second time constant was used to filter the photometer output before recording on a strip chart recorder.

A thin-layer plate spotted with a concentrated solution of the oxadiazole PBD was placed in position and used to provide a fluorescence signal while the optical components were aligned and focused.

The alignment procedure is critical for the TPEF and SEF techniques due to their quadratic dependence on laser power and inverse dependence on the cross-sectional area of the focused laser beam. Once optimized, the apparatus was found to be stable resulting in reproducible chromatograms. The vertical positioning of the thin-layer plate, in order to align a new sample with the laser beam, was the only adjustment that was necessary. The thin-layer plate was mounted on a small translation stage to facilitate this alignment.

Chromatography

Separations of various alkyl amines derivatized with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), and a mixture of oxadiazole compounds, were performed on reversed-phase, C_{18}, thin-layer plates (Supelco, Inc., Bellefonte, PA). The plates were cut to the dimensions of 3 x 20 cm and then prewashed to the top of the plate with methanol. This was followed by an additional washing in the development solvent. This washing procedure removed certain extraneous impurities, minimizing the fluorescence background of the plates. Sample volumes ranging from 200 to 500 nL were spotted in 50 nL increments using a 0.5 μL syringe. The spots were dryed between successive applications with either N_2 or air in an effort to minimize spot size.

The thin-layer plates spotted with the derivatized amines were allowed to develop to a distance of 10 cm from the origin in 50% HPLC

grade methanol in distilled water. The plates spotted with the oxadiazole mixture were developed to a distance of 12 cm from the origin in 75% HPLC grade methanol in distilled water.

CHAPTER II

RESULTS AND DISCUSSION

In contrast to elution chromatographic techniques, it is not necessary for the detection process in thin-layer chromatography to be in real time. Several different modes of detection can be performed in sequence, providing the detection methods are nondestructive. If the detection methods are selective, and yield unique spectroscopic information, it is possible to obtain quantitative and qualitative data for chromatographically unresolved components. The limited distance that thin-layer plates can be developed, and the lack of a simple and efficient means of altering retention parameters during the course of the development, generally leads to fraction capacities for thin-layer chromatography which are somewhat inferior to those obtained in liquid chromatography. This means that unresolved components are quite probable when thin-layer chromatography, even HPTLC, is used in the separation of relatively complicated mixtures.

The unique spectroscopic selectivities of the laser fluorometric techniques presented herein have been described previously (9, 25). The purpose of this work was to explore the potential of these techniques as detection methods for HPTLC. Sample thin-layer chromatograms illustrating the conventional fluorescence and SEF detection of several derivatized amines, and the TPEF detection of several oxadiazole compounds, appear in Figure 9.

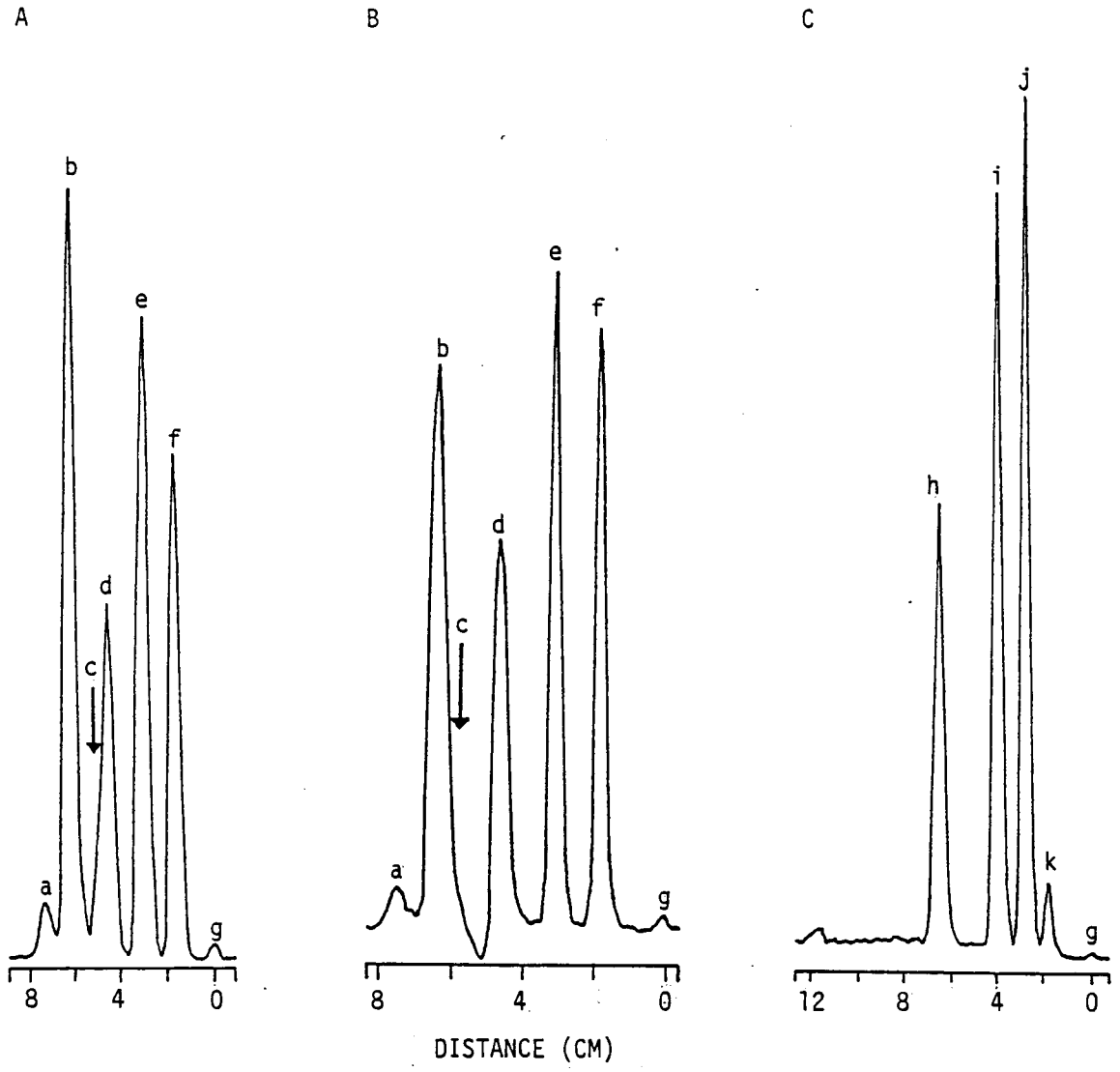

Figure 9. Chromatograms illustrating conventional fluorescence (A) and SEF (B) detection of several NBD amine derivatives, and TPEF (C) detection of several oxadiazole compounds. The chromatogram components are an unknown impurity (a), methylamine-NBD (b), excess NBD-Cl (c), dimethylamine-NBD (d), isobutylamine-NBD (e), isoamylamine-NBD (f), the origin (g), PPD (h), β-NPD (i), PBD (j), and BBO (k).

Sensitivity

The implementation of laser excitation sources in fluorometry results in increased signal levels. However, improvements in detectability may not be realized due to a concomitant increase in optical background levels. The specular scatter from thin-layer plates is extremely large when laser excitation is employed. This tends to minimize laser related improvements in detectability. Conversely, the static nature of thin-layer plates permits improvements in detectability through the employment of long detection time constants or signal averaging. The limits of detection (LOD) that we have observed for conventional fluorescence detection are in the low picogram range (see Table 1). This is approximately equal to the limits of detection typically observed for laser fluorometric detection in liquid chromatography.

The large blue shift between excitation and emission wavelengths for the TPEF and SEF techniques permits efficient spectral rejection of the intense specular scatter from the thin-layer plates. Optical background levels for these nonlinear techniques were near the dark currents of the PMT employed. Limits of detection, as seen in Table 1, were in the mid picogram range. This is a substantial improvement over that observed for TPEF and SEF detection in liquid chromatography. The limit of detection values shown in Table 1 were determined from calibration plots as the spotted amount that yielded a signal to noise ratio of two. The SEF limit of detection for the NBD derivative of

Table 1. Limits of Detection

Compound*	Technique	LOD (pg)
Methylamine	Conventional Fluorescence	9
Dimethylamine		32
Isobutylamine		15
Isoamylamine		18
Methylamine	SEF	70
Dimethylamine		-
Isobutylamine		90
Isoamylamine		110
PPD	TPEF	500
B-NPD		150
PBD		75
BBO		170

*Amine compounds are derivatized with NBD-Cl.

dimethylamine could not be determined due to interference from unresolved, excess NBD-Cl, present in the test mixture. The NBD-Cl has absorption bands that overlap the SEF emission of the amine derivatives.

Although the detectabilities demonstrated in this work are very good, improvements are expected with a few minor changes. The large background for the conventional fluorescence measurements was minimized by using both a monochromator and a sharp cutoff filter to isolate the emission. However, it was not possible to operate at the emission maxima for the derivatized amines due to the unavailability of a suitable cutoff filter. In addition, the bandpass filter arrangement used for SEF and TPEF detection transmitted only 20% at its maximum. Improvements in signal recovery would be expected with the employment of better filter arrangements. Fluorescent impurities on the thin-layer plates also adversely affected detectability, by increasing optical background and noise levels. These impurities, which appeared as small fluorescent specks when illuminated with a UV lamp, are thought to be carry-over contamination from the manufacturing of fluorophor-coated thin-layer plates.

The cross-sectional area of a focused laser beam has a quadratic dependence on the focal length of the lens employed (26). As previously mentioned, SEF and TPEF signals depend inversely on the cross-sectional area of the focused laser beam. Consequently, decreasing the focal length of the focusing lens should increase SEF and TPEF signal levels. This expected improvement was not observed. Only a

10% improvement in signal was observed when chromatograms were scanned using a 25 mm focal length lens instead of the 200 mm focal length lens, presumably due to surface or heating effects. More significant was the appearance of a large number of noise glitches. These glitches increase in number and magnitude as the cross-sectional area of the focused laser beam is made to approach the size of the fluorophor impurity specks.

Linearity

Calibration plots of NBD derivatized methylamine exhibited linear response from its limit of detection to 0.50 ng for conventional fluorescence, correlation coefficient of 0.9997, and to 2.0 ng for SEF, correlation coefficient of 0.9999. A TPEF calibration plot for the oxadiazole PBD exhibited a linear response from its limit of detection to 20 ng, with a correlation coefficient of 0.9948.

Detector saturation, during the intense laser pulse, and thermal problems limit the linear dynamic range for these detectors. Detector saturation can result from the large signal levels for the more concentrated samples. The large background from specular scatter in conventional fluorescence detection, also contributes to the detector saturation problem. This is evidenced by an earlier "roll-off" for the conventional fluorescence plot relative to the SEF plot. In both conventional fluorescence and SEF detection, the linear dynamic range was further limited by thermal effects, which occur when a large amount

of absorbed radiation is nonradiatively dissipated. The TPEF technique involves a very inefficient absorption process and, therefore, is not expected to be limited by these thermal effects. The linear dynamic range observed for the TPEF detection is more than an order of magnitude greater than that observed for the other techniques.

The laser fluorometric modes of detection investigated in this work yielded reproducible signals at low solute concentrations. However, at high concentrations slight reductions in signal were noticed during successive scans of the same thin-layer plate. These reductions in signal were absent for TPEF detection.

CHAPTER III

CONCLUSIONS

The feasibility of utilizing different modes of laser fluorometric detection in thin-layer chromatography has been demonstrated. The sensitivities of the laser excited fluorescence techniques were shown to be comparable to, and in some cases better than those obtained from commercial thin-layer fluorometric detectors. The excellent limits of detection observed for these techniques, and the unique focusing properties of the laser, should result in future applications of laser fluorimetry in HPTLC detection.

LIST OF REFERENCES

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1. Diebold, G. J.; Zare, R. N. Science 1977, 196, 1439.
2. Richardson, J. H.; Ando, M. E. Anal. Chem. 1977, 49, 955.
3. Yost, R. W.; Ettre, L. S.; Conlon, R. D. "Practical Liquid Chromatography - An Introduction"; Perkin-Elmer Corporation: Norwalk, CT, 1980.
4. Giering, L. P.; Horning, A. W.; Amer. Lab. 1977, 11, 113.
5. Yeung, E. S.; Sepaniak, M. J. Anal. Chem. 1980, 52, 1465A.
6. Richardson, J. H.; Larson, K. M.; Haugen, G. R.; Johnson, D. C.; Clarkson, J. E. Anal. Chem. Acta 1980, 116, 407.
7. Göppert-Mayer, M. Ann. Phys. 1931, 9, 273.
8. McClain, W. M. Acc. Chem. Res. 1974, 7, 199.
9. Sepaniak, M. J.; Yeung, E. S. Anal. Chem. 1977, 49, 1554.
10. Sepaniak, M. J. Ph.D. Dissertation, Iowa State University, Ames, Iowa, 1980, p. 123.
11. Lin, H. B.; Topp, M. R. Chem. Phys. Lett. 1977, 48, 251.
12. Frei, R. W.; Lawrence, J. F. "Chemical Derivatization in Liquid Chromatography"; Elsevier Scientific: New York, 1976.
13. Sepaniak, M. J.; Yeung, E. S. J. Chromatogr. 1981, 211, 95.
14. Lin, L. B.; Topp, M. R. Chem. Phys. Lett. 1977, 47, 442.
15. Novotny, M. Anal. Chem. 1981, 53, 1294A.
16. Scott, R. P. W.; Kucera, P. J. Chromatogr. 1979, 169, 51.
17. Kirkland, J. J.; Yan, W. W.; Stoklosa, K. J.; Dilkis, Jr., C. H. J. Chromatogr. Sci. 1977, 15, 303.
18. Knox, J.; Gilbert, M. J. J. Chromatogr. 1979, 186, 405.
19. Kasha, M. Faraday Soc. Discuss. 1950, 9, 14.
20. Zlatkis, A.; Kaiser, R. (eds.) "HPTLC High Performance Thin-Layer Chromatography"; Elsevier Scientific: Amsterdam, 1977.

21. Fried, B.; Sherma, J. "Thin-Layer Chromatography"; Marcel Dekker, Inc.: New York, 1982.

22. Berman, M. R.; Zare, R. N. Anal. Chem. 1975, 47, 1200.

23. Becking, M. K. L.; Kniseley, R. N.; Svec, H. J. Anal. Chem. 1983, 55, 200.

24. Bertsch, W.; Hara, S.; Kaiser, R.; Zlatkis, A. "Instrumental HPTLC"; Proceedings of the First International Symposium on Instrumentalized High Performance Thin-Layer Chromatography (HPTLC), Dürkheim, West Germany, Dr. Alfred Hüthig Verlag: New York, 1980.

25. Tromberg, B. J.; Eastham, J. F.; Sepaniak, M. H. Submitted to Appl. Spectrosc.

26. Wirth, M. J.; Kaskela, A.; Sanders, M. J. Appl. Spectrosc. 1981, 35, 14.

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