NEW DETECTION TECHNIQUES IN HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY AND RELATED STUDIES

by

Barry Todd Yeager

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CHEMISTRY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1995

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Barry Todd Yeager entitled New Detection Techniques in High Performance Thin Layer Chromatography and Related Studies

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Michael Burke	Date 11/6/95
Scott Saavedra	Date 11/6/95
Walter Miller	Date 11/6/95
William Salzman	Date 6 Nov 95

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Dissertation Director
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Date 11/17/95

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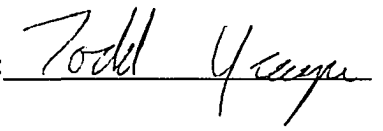
SIGNED: 

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For Ginger and Elizabeth,
with all my love.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

ABSTRACT

Recently, high performance thin-layer chromatography, or HPTLC has seen considerable growth as an analytical technique. Most often perceived as a semi-quantitative or preliminary technique, in reality HPTLC is an analytical technique in its own right. Modern instrumentation, improved stationary phases, and automated techniques have found their way into the realm of HPTLC, vastly improving the scope of the technique. Today, HPTLC is a very popular analytical technique, possessing many advantages such as ease of use, high throughput, high sensitivity, and low cost. HPTLC is also applicable to a wide variety of compounds in a wide variety of matrices. Despite these advantages, HPTLC has still not received the recognition it deserves as a true, quantitative analytical technique.

The following chapters will describe improvements in various stages of the technique. Chapter 1 will discuss the general theory behind HPTLC, highlighting its advantages and disadvantages, as well as areas needing improvement. Chapter 2 will discuss a new application technique in HPTLC that greatly improves upon current methodologies for sample application. Chapters 3 and 4 will discuss a novel detection technique used to image the entire plate simultaneously and give excellent quantitative information of analytes directly on the HPTLC plate. Chapter 5 will discuss the use of an infrared focal plane array as a detection technique that gives both qualitative and quantitative information. Finally, Chapter 6 will discuss the use of a CCD home video camera as an inexpensive array alternative to scientifically operated CCD's.

CHAPTER 1

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) was first referred to as a technique in 1938 by two Russian workers, Izmailov and Shraiber, who called the technique drop chromatography on horizontal thin layers.(1.1) Since then, thin layer chromatography has become an important analytical tool used not only by chemists, but also pharmacists, pharmacologists, biologists, and chemical manufacturers, to name but a few. Today, TLC can range in complexity from a simple high school lab experiment, to a highly advanced, instrumentalized technique. The flexibility of TLC makes it a very popular and very powerful technique applicable to a large number of different systems and separations. Figure 1.1 illustrates where most TLC based separations are being used today.

Thin layer chromatography is a separation technique in which uniform, thin-layers of a stationary phase, usually silica, are coated onto a support of suitable size to provide stability. Samples are applied to one end of the plate, several millimeters from the edge, and developed with a pure or mixture of solvents, known as the mobile phase. The applied spots migrate at differential rates down the plate, causing them to separate, based on their differential migration. Following separation, the mobile phase is rapidly dried off and the analytes are detected and visualized on the plate by a variety of methods. Each of these steps, application, theory and mechanism of separation, detection, and quantitation, will be discussed in this chapter, as well as the advantages and disadvantages of thin layer chromatography.

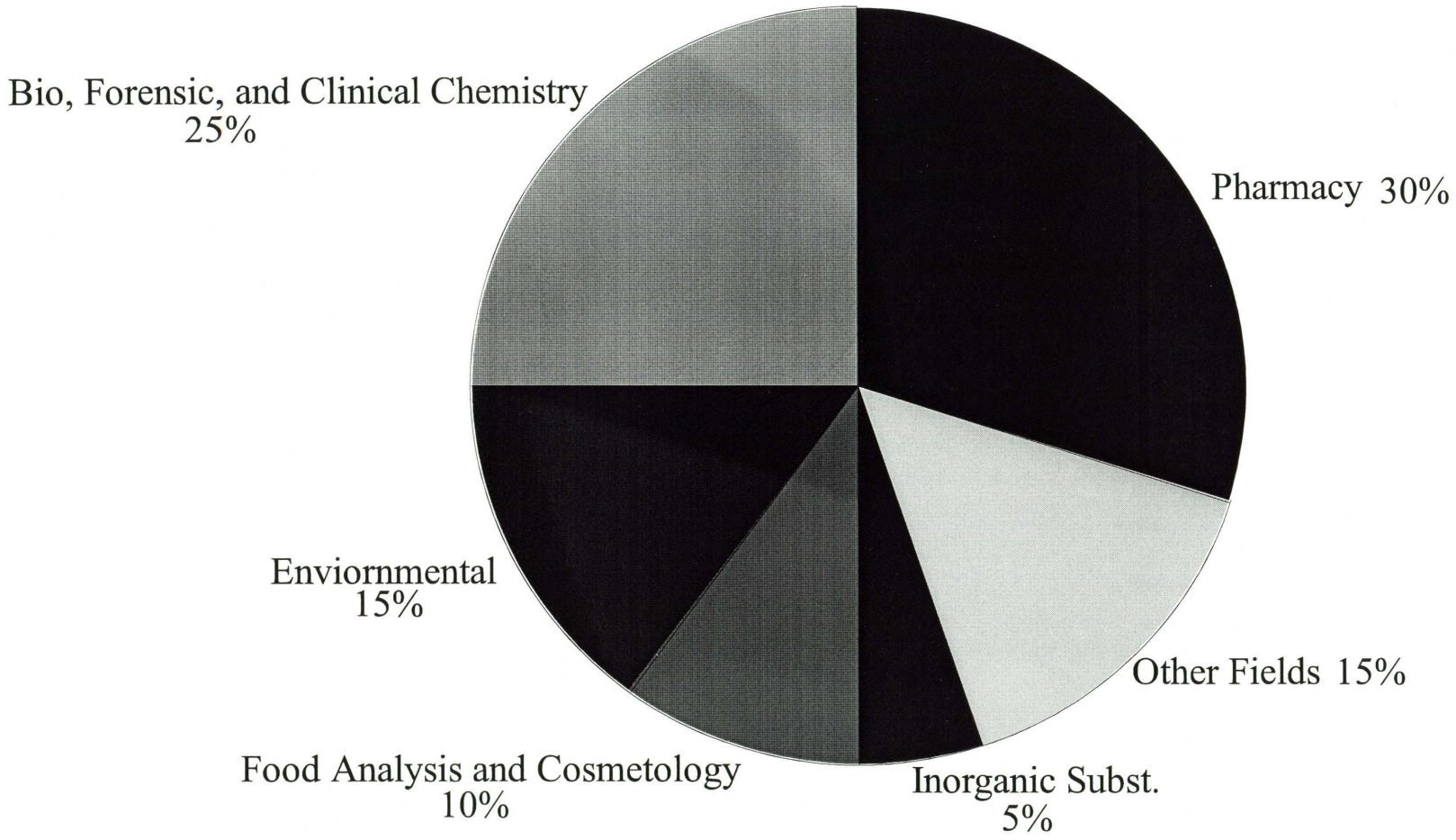


Figure 1.1, Fields of application for thin layer chromatography

Jork, Funk, Fischer, and Wimmer, Thin-Layer Chromatography, Reagents and Detection Methods, Vol. 1B

High Performance Thin Layer Chromatography

Most thin layer chromatographic based separations performed today are termed high performance thin layer chromatography. For thin layer chromatography to be considered high performance thin layer chromatography, certain requirements must be met. According to Kaiser(1.2), these requirements are:

- An optimized coating material with a separation power superior to the best HPLC separation material
- A new method for feeding the mobile phase
- A novel procedure for layer conditioning
- A considerably improved dosage method
- A competent data acquisition and processing system

These modifications, particularly those referring to changes in the stationary phase of the plates, are now nearly always incorporated into every TLC separation. Figure 1.2 illustrates the particle size distribution comparisons between high performance thin layer chromatography (HPTLC), thin layer chromatography (TLC), and preparative layer chromatography (PLC). The addition of these changes has helped make HPTLC a considerably improved and more quantitative analytical technique. These changes have contributed better detection limits, better separation ability, lower impurities of the stationary phase, shorter separation times, and the ability to run more samples on a single plate.(1.3) This has helped HPTLC become a separation technique in its own right, not just a semi-quantitative pre-cursor to performing an column-based separation.

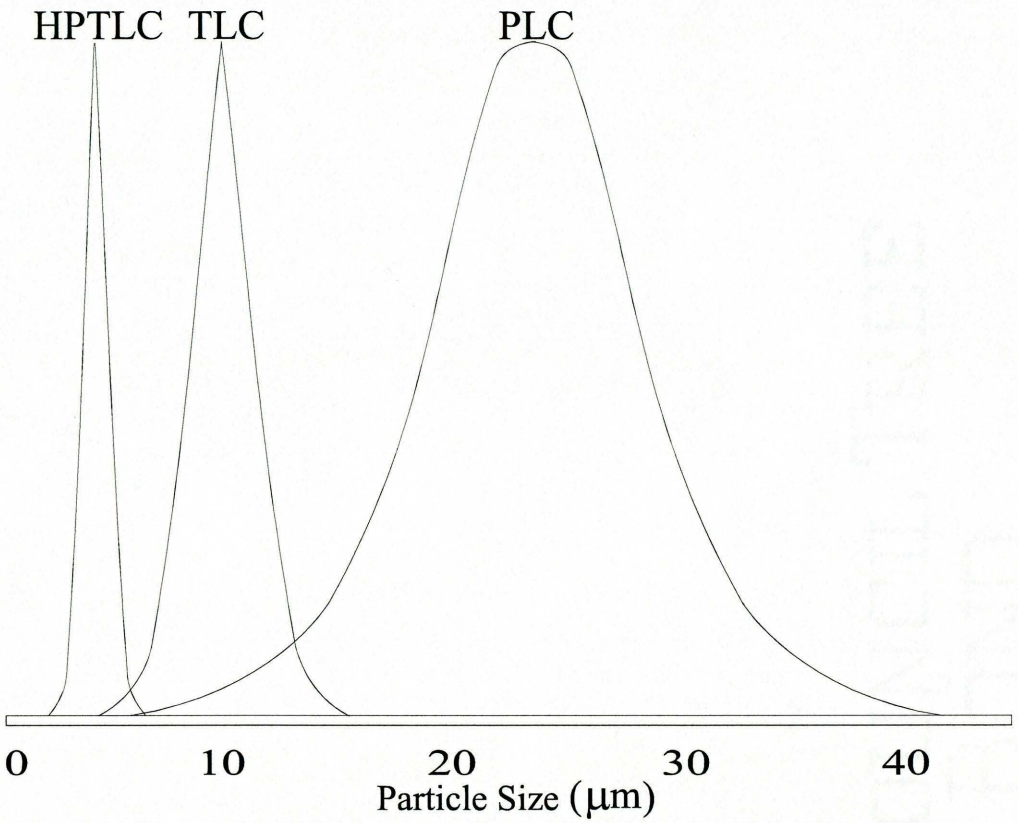


Figure 1.2, Typical particle size distributions of
High Performance Thin Layer Chromatography (HPTLC),
Thin Layer Chromatography (TLC), and Preparative Layer Chromatography (PLC)

Sorbents and Pre-Coated Layers in TLC, by H.E. Hauck, M. Mack, and W. Jost, in
Handbook of Thin Layer Chromatography, Chromatographic Science Series, Vol.55
Sherma, J. and Fried, B. eds. Marcel Dekker (1991).

Sample Application

Sample application is a very important step in performing a successful HPTLC separation. If the sample is improperly applied, the desired degree of separation may not occur. There are certain requirements a technique must have to be used as a HPTLC application system. The requirements are small spot size, high accuracy, and high precision. Sample application should also be simple and as rapid as possible. Ideally, the application technique would not disturb the separation media. The system should also be automatable and inexpensive. There are a variety of ways to apply a sample to the plate, including microcaps, glass capillaries, microsyringes, and fully-automated application systems, and these methods are more thoroughly discussed in Chapter 2.

Theory and Mechanism

To understand the separation mechanism of HPTLC, one must understand the flow of the liquid phase. The flow in HPTLC, unlike HPLC, cannot be controlled without the use of special equipment, and this equipment is not commonly used. The flow is dependent on the surface tension and viscosity of the mobile phase, and the nature of the stationary phase. There are many models to try to predict the flow of the mobile phase. One of these models considers the stationary phase to be composed of interconnected capillaries of varying diameter.(1.4) Initially, the stationary phase is dry. As the liquid mobile phase is applied at one end, it is drawn up into the stationary phase by capillary action. A homogeneous bed is preferred to minimize variations in capillary flow across the bed. However, since the bed is not perfectly homogenous, the quantity of liquid on the bed varies, with most of the mobile phase present at the origin. The distance

the solvent front moves, s_f has been shown to be proportional to the square root of the migration time, t, according to the following equation:

$$s_f=\sqrt{(kt)}$$

where k is a proportionality constant, directly proportional to the surface tension and diameter of the particle, and inversely proportional to the viscosity. The velocity of the solvent front, U_f, is defined as follows:

$$U_f=k/2s_f$$

and is proportional to the surface tension of the mobile phase and inversely proportional to its viscosity, and the distance the front has moved. As stated earlier, the solvent velocity is not constant and decreases the further away from the origin it has migrated. Eventually, the solvent velocity, for ascending methods, decreases to zero. This puts an upper limit on the distance the solvent can migrate and limits the effective size of the HPTLC plates. This can be overcome somewhat by using smaller and more uniform particle sizes, but an efficiency limit is reached at about 3 μm particles, where the price increases dramatically, but increased separation efficiency is not clear.(1.5) With the incorporation of these smaller particles, typically from 5-6 μm in diameter, the number of theoretical plates has risen dramatically, from a few hundred, to over several thousand. Separations can also be performed faster and in about half the distance required by the plates with the larger particles.(1.6) It should be noted that HPTLC separations do not yet match the separation power of HPLC separations, but improvements in layer

homogeneity and particle size distributions will eventually bring HPTLC separations up to the separation ability of column-based separations.

Detection

There are many different methods for detecting components on a HPTLC plate, ranging from visualization with the eye, to employing expensive densitometric detection systems. The wavelength range that can be used by various methods to detect the analytes on a plate extend from the Ultraviolet to the Mid-Infrared regions. One of the advantages of detecting analytes on a HPTLC medium as opposed to a column-based medium is static detection. This means that the analytes are immobilized on the plate and can be detected with a variety of different methods and repeatedly scanned if desired. In a column medium, the analytes are evaluated typically only once, as they migrate past the detector, and many aliquots must be collected if the sample is to be analyzed later by a different method. Additionally, since HPTLC plates use static detection, the plates can be efficiently stored and reanalyzed at a later date if the circumstances require it.

The simplest method for detecting analytes on the HPTLC plate is to use visual inspection. This method requires that the spots absorb light in the visual portion of the spectrum, so they can be seen with the eye. If the spots do not natively absorb in the visible, steps must be taken to visualize the spots so they can be detected. This involves the use of visualizing reagents that causes the spots to appear through a variety of methods. The most simple and common visualization method is the use of iodine crystals. The developed plate is placed in a chamber containing a small amount of crystalline iodine. After a short period of time, the iodine vapor binds to the analytes, causing them to appear brown to the eye. Another visualization method involves the use

of chemicals sprayed or dipped onto the plate to chemically react with the spots to give them an absorbance in the visible. These reagents are often specific for only for one class of compound, causing other analytes to go undetected.

Another method for detecting analytes on the plate involves the use of instruments that measure absorbance. Visible absorbance can be used, but only if the analyte of interest has a native visible absorbance or has been treated to make it visible. The most common type of absorbance used is UV absorbance, often called fluorescence quenching. UV absorbance is performed on HPTLC plates specially coated with an inorganic fluorescent dye. The dye fluoresces in the visible (typically green) region of the spectrum when illuminated with UV light, usually at 254 nm. The principle of detection is that analytes on the plate absorb a portion of the ultraviolet light that causes the plate to fluoresce, causing the analyte to appear as dark spots on a fluorescent background. The more analyte present, the darker the spot appears. Using this technique, any analyte that has an absorbance at 254 nm will be detected. The closer to 254 nm the absorbance maximum of the analyte of interest is, the more sensitive the technique. Consequently, if the absorbance of the compound at 254 nm is small, the sensitivity and effectiveness of the technique diminishes. Overall, UV absorbance is an excellent all-purpose detection method that is applicable to a large number of separations.

Another way of detecting analytes on the plate is the use of fluorescence. This technique operates by illuminating the plate with UV radiation that causes the analytes of interest on the plate to fluoresce. A filter is typically used to block the UV illumination from reaching the detector. Fluorescent intensity is directly proportional to amount of analyte present on the plate. This method works only if the analyte of interest has enough native fluorescence to be detected, or has been derivatized by some means to give the compound fluorescent abilities.

Quantitation

The most popular means of spot quantitation used today is the scanning densitometer. The scanning densitometer operates by rastering the separation lanes with a light beam in the form of an adjustable slit. The difference between the optical signal from the sample-free background and the signal from the analyte spots corresponds to the amount of analyte on the plate. Scanning densitometry is a sequential technique, meaning the instrument scans a plate lane-by-lane, and the spots are detected one at a time. The instrument can employ single or double-beam geometry, and can be operated in reflectance or transmittance mode using absorbance or fluorescence detection. While providing good quantitative results, the technique is slow and reduces the throughput of HPTLC, its main advantage, to the level of linear techniques, since each spot must be individually read out. A typical system configuration of a scanning densitometer is shown in Figure 1.3.

Another method of quantitation involves the use of a charge-coupled device (CCD). A CCD is a two-dimensional multi-channel detector that is perfectly suited to the HPTLC format. In essence, a picture of the plate is taken and the area under the peaks, regardless of peak shape, can be integrated. This is done in a fraction of the time it takes a scanning densitometer to read out the plate one lane at a time. This allows for the high throughput of HPTLC to be exploited to its maximum advantage, since detection of the analytes is no longer the limiting step. The CCD, like the scanning densitometer, can be run in both reflectance and transmittance modes, and can employ absorbance or fluorescence. The operation of the CCD is more fully described in Chapter 3.

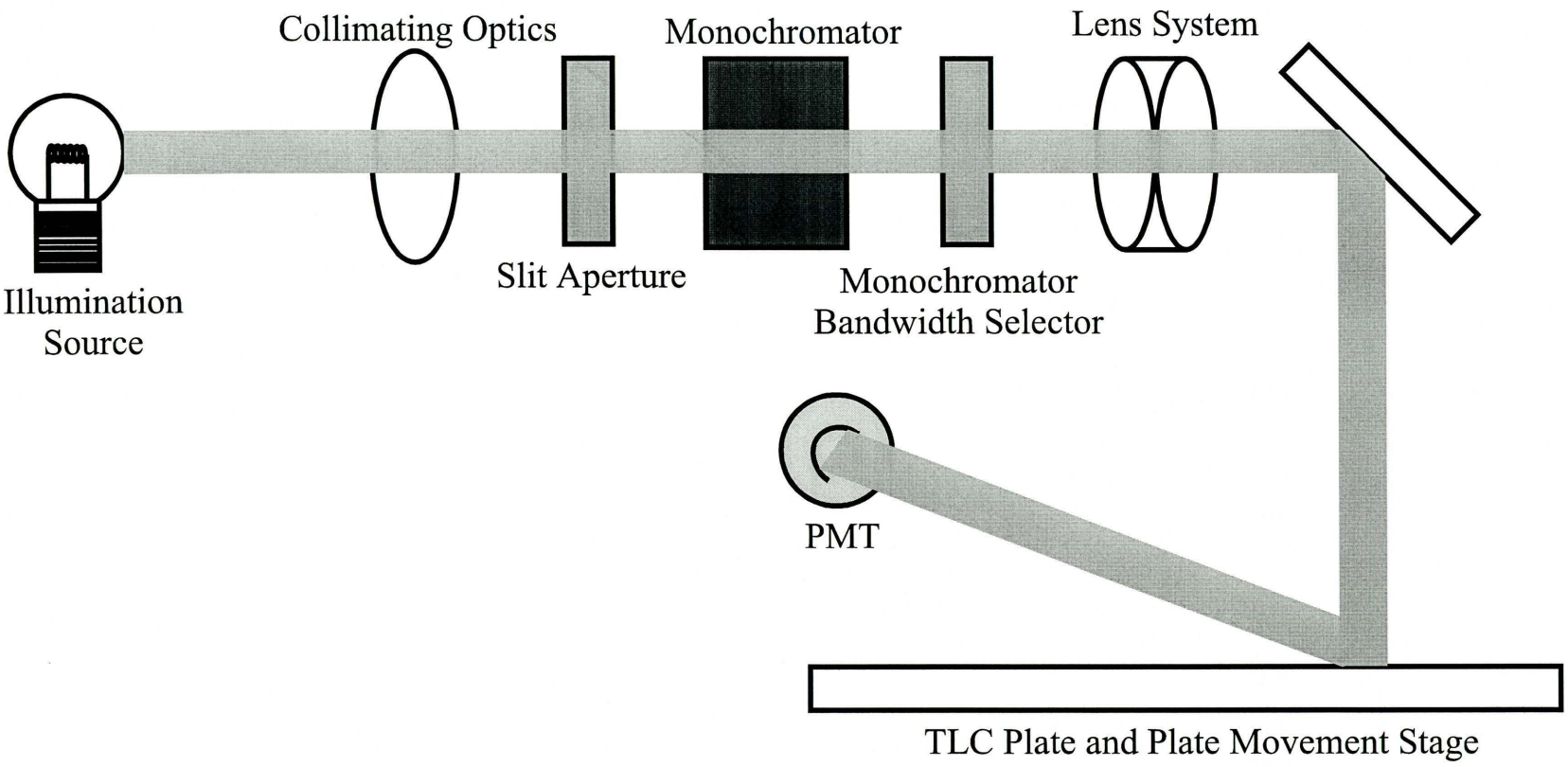


Figure 1.3, Scanning densitometer system configuration, based on CAMAG TLC Scanner II

Advantages/Disadvantages

There are many advantages to performing an analysis on a HPTLC medium. The main advantage of using HPTLC is throughput. The parallel nature of the separation medium allows for many samples to be separated simultaneously in the time that it takes for a column-based medium to perform just one. This advantage is particularly important in routine monitoring of contaminants and by-products, where a large number of representative samples must be taken to ensure true sample representation. This large throughput ability also enables standards to be run on the same plate with the unknowns, reducing the number of total separations required. Another advantage of HPTLC is the wide variety of stationary and mobile phases available to help achieve optimum separation. The stationary phase can consist of silica, cellulose, alumina, magnesium oxide, magnesium silicate, powdered glass, polyamides, or mixed-layer stationary phases, which contain a combination of two or more of these layers. This large variety of layers almost guarantees that a suitable stationary phase can be found to perform the desired separation. Mobile phase consumption in HPTLC is low, reducing the cost of each separation as well as reducing waste disposal. Low cost per separation is another advantage HPTLC enjoys. Expensive instrumentation is not required to perform the separation, and many samples can be run on a single plate to maximize savings. Another important advantage in HPTLC is that every sample is analyzed on a fresh stationary phase each time. There is no danger of unforeseen residues from previous samples contaminating the run, as is possible in a column-based medium. Non-migrating analytes in a column medium can bleed off slowly over time, or never come off the column, possibly altering future separations and results. Since the HPTLC plate is disposable, sample preparation requirements are not as stringent as with other separation schemes and less pure samples can be applied without fear of contaminating the medium. This also

cuts down on operator time in preparation of the samples, which realizes further savings in operating costs. HPTLC also has the added advantage of total sample accountability, meaning that non-migrating species will be easily seen at the origin. This is important when one must account for all compounds and by-products in a reaction to understand which reactions have taken place. This ability is particularly important in the pharmaceutical field, where an undetected compound in a particular synthesis may have harmful side effects. In a HPTLC medium, all analytes, whether they migrate or not, are detected and accounted for. This is not possible in a column medium, with analytes only being detected if they migrate down the column to the detector. Another advantage of HPTLC is static detection, which means the separated analytes to be detected are immobilized on the plate rather than eluting off a column. This means the plates can be scanned repeatedly using different parameters, or can be stored and later retrieved for further analysis without having to worry about collecting and storing many separate samples eluted off a column. Detection in HPTLC is not limited by the choice of mobile phase required to perform the separation. Since the mobile phase is dried off before the analysis is performed, highly fluorescent mobile phases can be used to separate weakly fluorescing analytes. This is not possible in a column medium, since the detector looks at the analyte still in the presence of the mobile phase. The separation power of HPTLC can be further increased by implementation of two-dimensional development. This involves spotting the analytes on one corner of the plate and developing them in one direction, as in a normal separation. The plate is then rotated 90 degrees and the plate developed again with a different solvent. While the throughput advantage is lost, the separation power can be greatly increased. This increases the range of separations that can be performed on an HPTLC medium. These features help make HPTLC an attractive alternative to column chromatographic techniques.

The disadvantages to using a HPTLC medium primarily arise from the lack of separating power required for some of the more difficult separations. Compared to column-based media, HPTLC has a more limited separation power and thus is not always applicable to a particular separation. Another problem associated with HPTLC is the uneven flow encountered when developing a plate. The uneven flow can adversely effect a separation to some extent and becomes a problem in difficult separations. This problem can be reduced to some extent with forced-flow development chambers, but that solution is an added cost to a system that is often chosen for its low initial cost. Air-sensitive analytes can often be a problem for an HPTLC separation, since the analytes are exposed to an open system. This can be avoided to some extent by the use of a glove bag or glove box containing an inert atmosphere, but this additional step is inconvenient at best. Another disadvantage is that temperature gradients can exist across the plate. Since the adsorption of liquid at the solvent front on the stationary phase is exothermic, this causes the temperature near the solvent front to be greater than its surroundings(1.7). This can cause partial distillation of a mobile phase that is a mixture of solvents, changing the properties of the mobile phase as it migrates along the plate. Another "problem" with HPTLC is the perceived lack of quantitative information available after the separation. In reality this perception is simply not true. This perceived problem is often a deciding factor when choosing between HPLC and HPTLC. Finally, to obtain quantitative information from a separation, additional equipment is required to analyze the plate. This additional equipment is a considerable increase in cost over the relatively inexpensive separation system. The cost of the typical quantitation system, the scanning densitometer, places the cost of the entire HPTLC system at about the same level as a column-based HPLC system. This fact, combined with the perceived limitations of only semi-quantitative results, compels many to choose a column based separation system

over a HPTLC based system. These limitations, particularly in the detection and quantitation areas of HPTLC, will be addressed in later chapters of this work.

Conclusions

High performance thin layer chromatography is an excellent separation technique with many features that make it an ideal choice for many separations in many different fields. The combination of low cost, excellent throughput, good separation ability, disposable media, and static detection helps make HPTLC a viable separation technique in its own right.

CHAPTER 2

INVESTIGATIONS INTO THE USE OF A MICRO-NEBULIZATION NEEDLE FOR SAMPLE APPLICATION IN HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

Background

One of the major requirements for thin layer chromatography (TLC) to be high performance thin layer chromatography (HPTLC) is that application zones be as small as possible.(2.1) Sample application is a very important aspect of a successful HPTLC separation. Sample application is the transfer of analyte in dissolved form to the silica surface of the HPTLC plate. This process can determine whether or not the desired separation will occur or not.

In a HPTLC separation, the analyte of interest is dissolved in a suitable solvent, and applied to the plate in amounts typically ranging from 1 to 10 microliters. Application amounts vary due to the amount of analyte in solution, and more specifically, the detection limit or desired amount of analyte on the plate. If the amount of analyte applied to the plate is too low, the analyte will not be detected. Conversely, if the amount of analyte applied to the plate is too high, the result is poor separation caused by the plate being overloaded. This causes irregularly shaped peaks, streaking, and/or tailing. Trial and error is often needed to get the analyte concentrations in the appropriate amounts on the HPTLC plate.

The selection of a solvent for application of analyte to the HPTLC plate is a critical step for achieving reproducible chromatography with distortion free zones. The

application solvent should completely dissolve the analyte and should be reasonably volatile, as well as weak in relation to chromatographic properties. High volatility is important to facilitate solvent evaporation on application of the spot. If the solvent is not volatile, evaporation of the applicator solvent may be incomplete, which could interfere with the developing solvent and cause distorted zones.(2.2) Additionally, if the analyte migrates with the application solvent, then the analyte will migrate as the spot is being applied, causing larger, donut-shaped rings, also causing distorted zones.(2.3) Ideally, the chromatographic surface should not be touched during the application process. Touching the surface of the silica disturbs the separation ability of the surface, causing distortions in the separation.(2.4)

There are a wide variety of methods to apply an analyte to a HPTLC plate. These methods range from using inexpensive glass capillaries to complete application systems costing upwards of $20,000 dollars. The most inexpensive method is to use disposable glass capillaries. These capillaries are dipped in the solution containing the analyte or mixture of analytes. This causes the solution to draw up by capillary action. The tip is then pressed on the surface of the plate and the solution is applied to the plate. This is typically done several times, allowing the solvent to evaporate before the next application. This is done for all spots on the plate. The method leaves much to be desired. This method is not quantitative and is only used for qualitative analysis or perhaps as a preliminary step to determine separation ability. In addition to the lack of quantitative information available, the application process involves touching, and often applying pressure to the plate. As previously mentioned, this adversely effects the separation of the analyte. Additionally, the analyte goes down in solution, causing the spots to be larger than desired. An alternative method to glass capillaries is the use of Drummond Microcaps. Microcaps are available in a wide variety of sizes, ranging from

0.25 to 100 microliters. The microcaps are operated by inserting disposable glass capillary tubes into the septum of a support tube. They are filled with solution and touched to the surface of the plate, where the solution is drawn out. If the solution is not completely expelled, a rubber bulb is squeezed to apply the entire contents of the capillary. This method is more quantitative than the use of capillaries alone and more precise. However, the surface of the silica is still touched, adversely affecting chromatography. Another method for applying analytes is the use of microsyringes. Use of a microsyringe involves filling it with the solution of interest and touching it to the plate so that the solution may be dispensed. This process is often automated and the procedure brought under computer control. Precision of application is a function of the precision of the micropipet, which is typically around 5%(1.8). High-end systems typically employ micropipets which allows variable amounts of solution to be applied to the plate, an advantage of the use of micropipets. However, the sample is still applied by physically touching the plate. In addition, the sample is applied "wet" to the plate. This requires that the application solvent be weak relative to the chromatographic properties of the analyte. Ideally, a sample applicator would have the ability to apply the sample to the plate in "semi-dry" form without touching the surface of the plate and have excellent accuracy and precision. This chapter discusses the development of a system to perform this task.

Original System

Sample applicator

The original system designed to apply samples to the plate utilizes a Harvard Syringe Infusion pump (Harvard Apparatus, Model 22) with a 50 microliter syringe and a three-way valve for system purging, coupled to a Rheodyne HPLC sample injector

(Rheodyne, Model 7520) with a 0.5 microliter loop. The pump is run at 4.00 microliters/minute. A 25 microliter syringe is used to load the injection loop which delivers a precise quantity of analyte into the specially designed micro-nebulizer. The applicator system is diagrammed in Figure 2.1. The nebulization head uses a modified HPLC injection needle (26 gauge, .0195" O.D., .0045" I.D.) with a tip cut flush with the end of the outer nylon sheathing of the nebulizer. The inner tube of the nebulizer was utilized for sample delivery and was made from a stainless steel syringe needle tip which had been cut to an approximate length of 16 mm. The needle is symmetrically sheathed with a nylon head to allow for nitrogen gas to be delivered around the needle which allows the analyte to be sprayed down in a controlled fashion. The nebulizer tip was mounted approximately .1" from the chromatographic surface during application and was connected to the injector with .005" I.D. (5 cm length) tubing to minimize dead volume. The mobile phase utilized for sample application was reagent grade methanol. The nebulizer head is shown in Figure 2.2. Deposition times were 4 minutes at an application rate of 4 μL/min. and a regulator pressure of 28 psi. Spot diameters were approximately 3 mm and ring shaped. Glass-backed Macherey-Nagel HPTLC plates (Macherey-Nagel Nano-Sil 10) with 100 μm layer thickness were used for the separation process. The analyte used for analysis of spotter precision and accuracy was aflatoxin G1, applied as 515 picogram spots.

Illumination and Imaging System

Illumination was accomplished by using a specially constructed transilluminator. The transilluminator consists of two 8 watt, long wavelength (365 nm) UV bulbs (Spectronics Corp. BLE 8T365) mounted within an aluminum housing with a wide

bandpass UV filter (Hoya Optics, U-340) mounted over a hole cut in the housing. Plates were placed silica side down in direct contact with the UV pass filter. Spectral selection was performed with Kodak Wratten filters #2E and #2B mounted on the front of the camera lens. Flat-fielding was performed using a separate fluorescent filter (Kodak, Y-48 fluorescent filter) to map spatial UV illumination differences. Flat-fielding is discussed in Appendix A. The camera system utilized in this study was a PM512 (Photometrics, Ltd. Tucson, AZ) scientifically operated CCD, cryogenically cooled to -100°C to reduce dark current. The system is diagrammed in Figure 2.3. Operation of the CCD camera is more thoroughly discussed in Chapter 3.

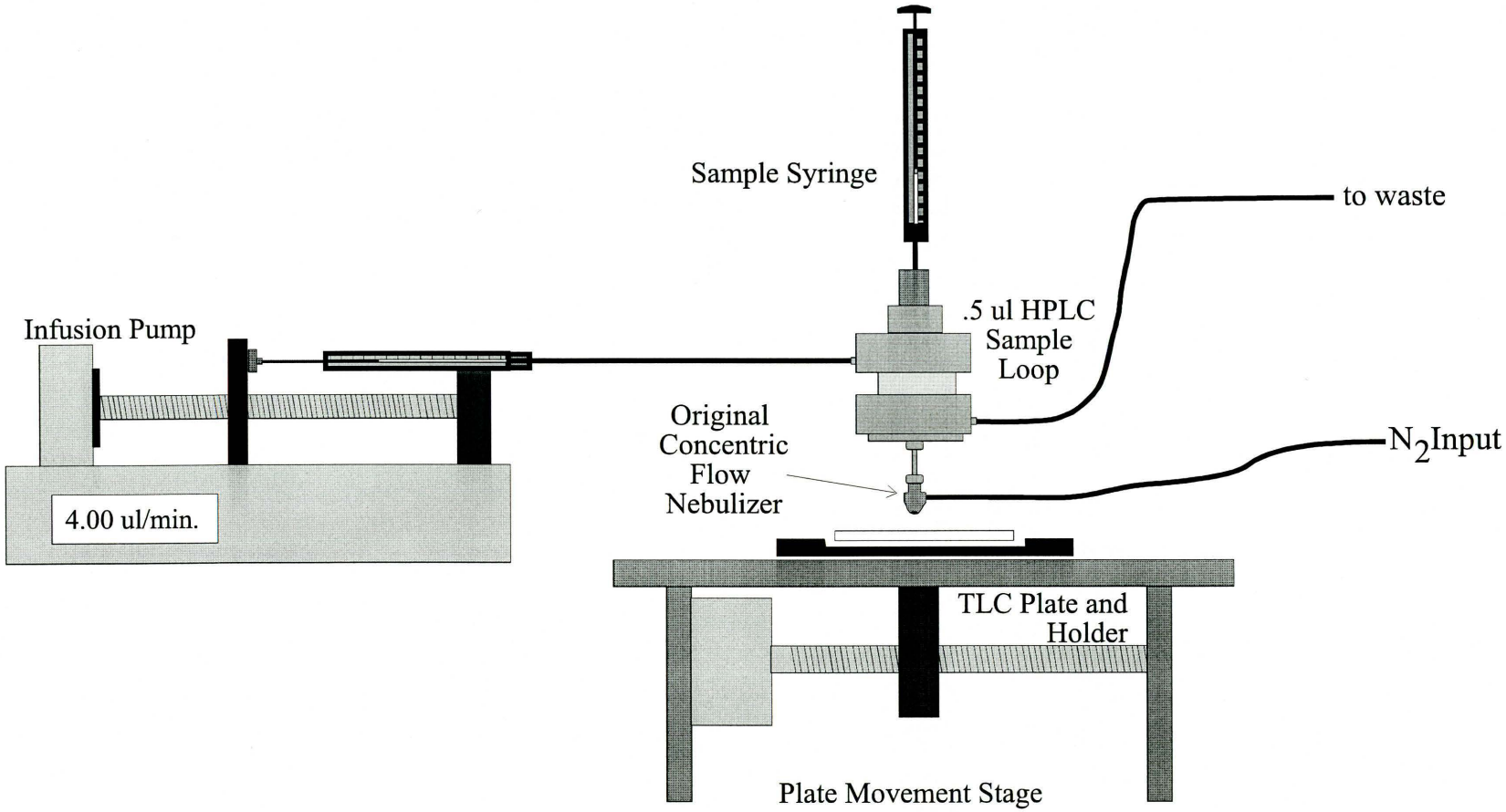


Figure 2.1, Original sample applicator system

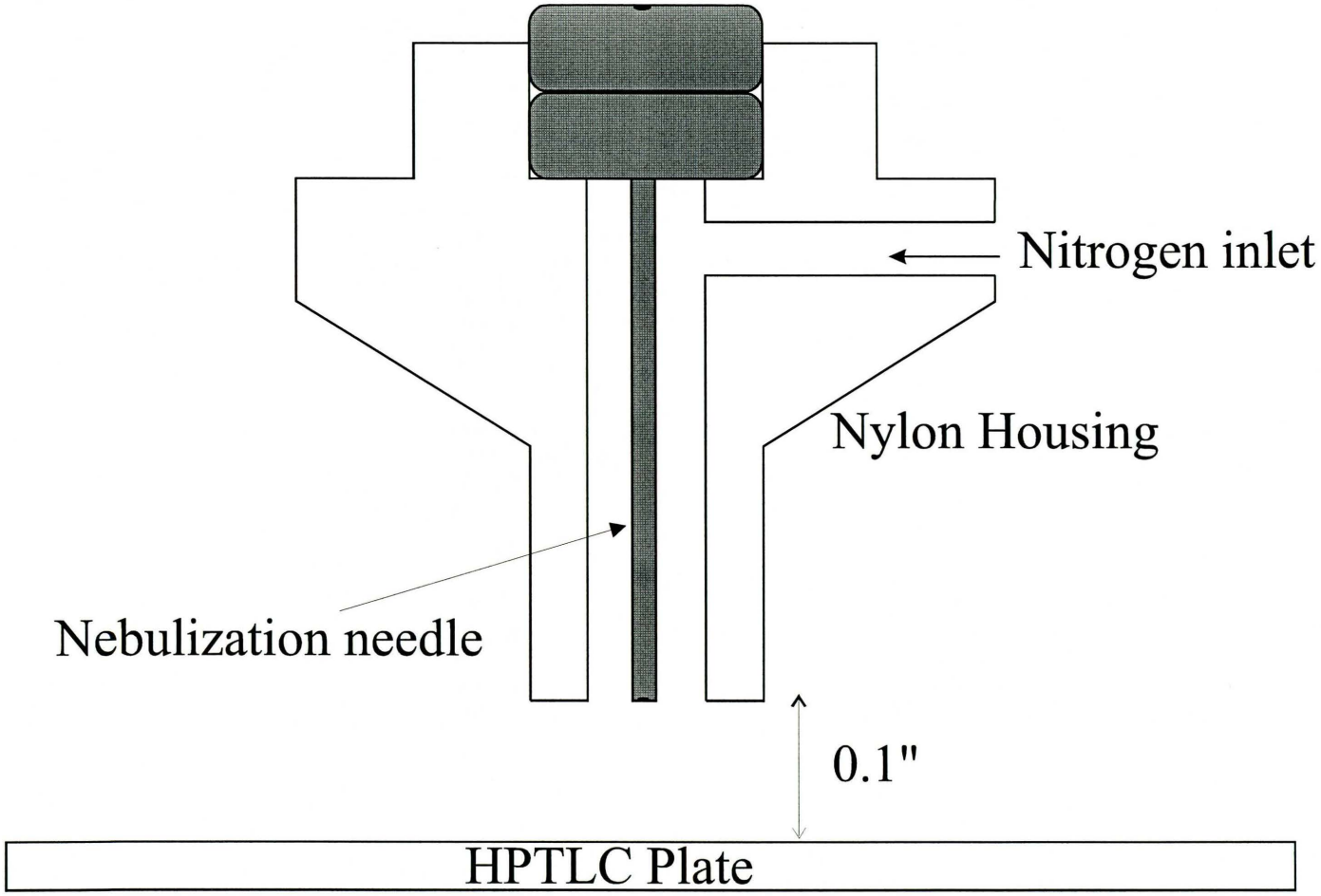


Figure 2.2, Original Micro-Nebulizer Head Design

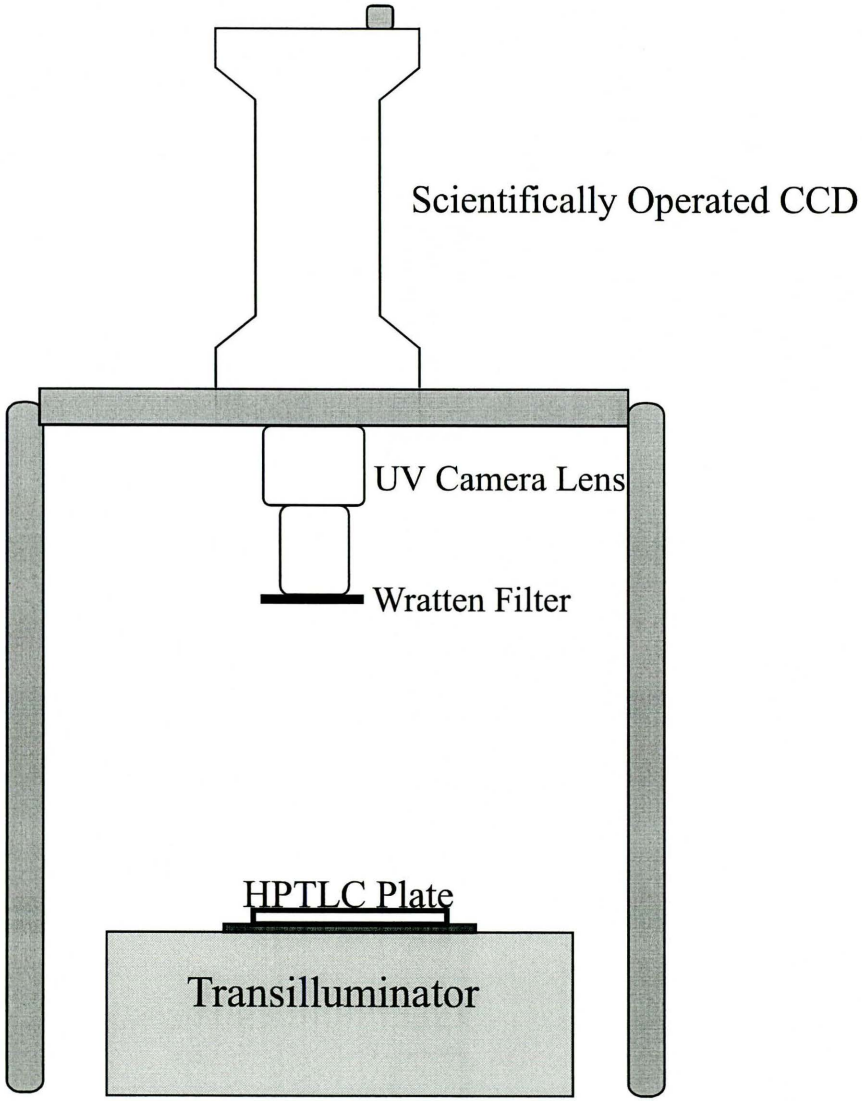


Figure 2.3, Original system detector configuration

Results

Precision. In order to evaluate system precision, replicate 515 picogram samples of aflatoxin G1 were spotted onto the chromatographic plate. The integrated fluorescence observed with the scientific CCD camera of the samples was measured before chromatography to eliminate added imprecision arising from the chromatographic process. All images were bias and flat field corrected. The results of the inter-spot precision are shown in table 1. Spot intensities were integrated using SpectraCalc (Galactic Industries). Results from the trials were as follows:

Trial	# of Samples	%RSD
1	3	3.9
2	3	2.0
3	3	1.5
4	4	1.4
5	4	0.8
6	4	2.7
7	4	4.0
8	4	3.5
9	4	1.5
	Pooled %RSD	2.4

Table 2.1: Original System precision results.

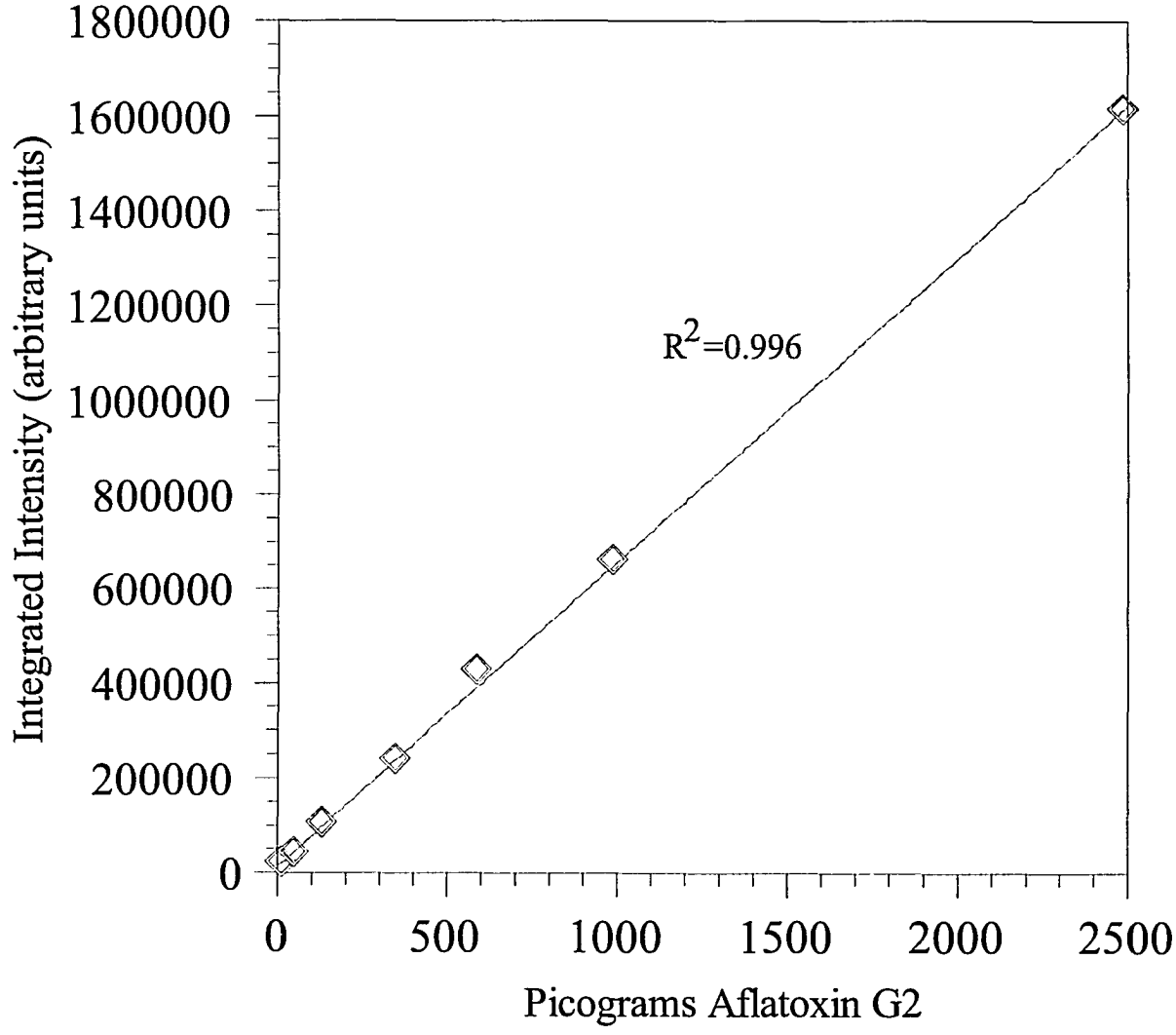


Figure 2.4, Original system accuracy

Accuracy: To determine accuracy of the sample applicator, varying concentrations of aflatoxin G1 were applied to the plate, bias corrected, flat fielded with the fluorescent filter, and integrated. The system shows excellent linearity as shown in Figure 2.4, with a linearity of 0.99.

Discussion

The system showed good accuracy and precision but improvements need to be made in several areas. The precision data is somewhat flawed, in that occasionally a data set will have a population of only three instead of four. This was caused by the presence of a solvent bubble forming on the needle tip and dropping off onto the silica surface, ruining the spot. The bubble was always present during sample application and was sensitive to system vibration and shocks, and when they occurred, the solvent bubble would vibrate and drop onto the spot being applied. This caused an unacceptable number of spots to be ruined. Additionally, spot size was larger than desired, with spots being approximately 3 mm large and ring shaped. The size and shape of the spot is a function of the shape of the nebulizer needle tip. Since the tip was flat, this caused the solvent bubble to form, and this in turn caused the spots to be large and ring shaped. The nitrogen gas would flow past the needle and bubble, picking up and vaporizing only the solvent along a ring on the outside of the solvent bubble. This caused the spots to be applied as rings and in a larger size than desired. This also caused deposition times for each spot to be longer, since the analyte being applied was "trapped" in the solvent bubble, requiring additional deposition time for each spot to ensure that all of the analyte

was out of the bubble. These areas are the primary focus of the redesign effort for improving the performance of the applicator.

The next generation of sample applicator was designed to eliminate flaws in the first generation. Primary focus for redesign was the applicator head and needle, but design improvements were also made in nitrogen gas delivery temperature and better determination of system results.

Modified Sample Applicator

System Setup

The system setup was essentially the same as the original system with several important modifications designed to increase the accuracy and precision of the unit. The main modification made was the redesign of the nebulizer head and needle. As previously mentioned, the flush cut of the nebulization needle caused a "solvent bubble" to form on the end of the needle. To alleviate this problem, a "sharper" needle was needed to eliminate the solvent bubble and the problems associated with it. Physically sharpening the needle caused imperfections to appear on the otherwise polished needle surface. These "micro-channels" caused the solvent to be drawn out by capillary action and drawn up the needle surface where the analyte was dried on the surface by the nitrogen gas, rather than being applied to the plate. This occurred to some extent even when the needle was polymer coated. This problem was eliminated by the use of a specially constructed needle constructed by Unimetrics. (Unimetrics, Shorewood, IL.) The needle was a 26s gauge with an 0.006" inner diameter and an 0.019" outer diameter. The needle was symmetrically sharpened and polished by Unimetrics so there were no surface imperfections to cause the solvent to be drawn up the needle. The needle was mounted in a newly constructed nebulization head. The nebulizer head is shown in

Figure 2.6. The head was redesigned to have an adjustable distance between the tip of the needle and the tip of the nebulizer head. This was done so that the best possible spray profile could be found to deliver the best spot shape and profile. To facilitate sample evaporation as the spot is being applied, a heat source was added to the nitrogen gas inlet tube. Heating tape was coiled around the metal tubing that delivers nitrogen into the nebulization head. The heating tape is plugged into a Variac current control source to vary the heat to the desired level of additional solvent evaporation to obtain the best possible applied spot. The modified system is shown in Figure 2.5.

An additional change was made to better detect the changes that improvements in the sample applicator would deliver. The quality of the HPTLC plates was improved by using 5x5 cm, 200 micron thick silica Merck plates (Merck, Darmstadt, Germany) which appear to have the best layer homogeneity and lowest localized fluorescent impurities. The spectral filter was also changed from the Kodak Wratten filters to a 2" float glass CVI brand filter (CVI Laser Corporation, LWPS-400, Albuquerque, NM.). The filter was mounted between the shutter and lens to reduce surface reflections that occurred between the glass plate and the filters that added noise to the system. This also eliminated a source of fluorescence that was emanating from the Kodak filters. The plate holder was coated with black velvet to reduce stray light coming from reflections off the aluminum case. The entire detection system was encased in light tight cardboard commonly used by photographers to block out unwanted sources of light emanating from various sources in the room.

An additional change for the analysis of spotter precision and accuracy is changing the way the spots are analyzed on the plate. Previously, fluorescence was used to determine precision of applied spots. There are several problems associated with using fluorescence. The first problem arises from using a separate fluorescent flat field filter to

map UV source intensity. The problem arises from the fact that all HPTLC plates vary from point-to-point and these differences affect the intensity of the ultraviolet light striking the plate. The flat field fluorescent filter can map spatial source intensities but not how these spatial intensities are transmitted through the plate. Variations in layer thickness of the silica on the plate are not compensated for, nor are impurities distributed unevenly across the plate. These variations can adversely affect measurements made of spot precision and accuracy and make the data unsatisfactory. The plate itself can be used as a flat field, but very long exposures must be taken, on the order of 5 minutes or more to get the intensities up to a detectable level. Using this method, localized fluorescent impurities can be somewhat corrected for by the flat field process, but plate repositioning is often not precise and the correction is not entirely successful. Additionally, if the developed spot lies on one of the localized impurities, it is impossible to get an accurate reading for the intensity of that spot.

An alternative method that does not require long exposures to obtain a flat field is the use of UV absorbance, commonly known as fluorescence quenching. In UV absorbance, the plate (Merck, F_{254} , 5x5 cm pre-scored, 100 micron thick, Art. 5635) is precoated at the factory with a fluorescent compound that fluoresces green when excited by 254 nm light. When a compound is present on the plate, it absorbs some of the UV light proportional to the amount of analyte and appears as a dark spot against a fluorescent background. This fluorescent background allows the operator to flat field the background and correct for spatial source intensity, plate inhomogeneity, and layer thickness inhomogeneity prior to spots being applied to the plate.. Essentially, this allows a "before-and-after" image of the plate and allows for superior image correction. A mercury vapor lamp was used to illuminate the plate from above the plate in reflection mode. The detector system setup is shown in Figure 2.7.

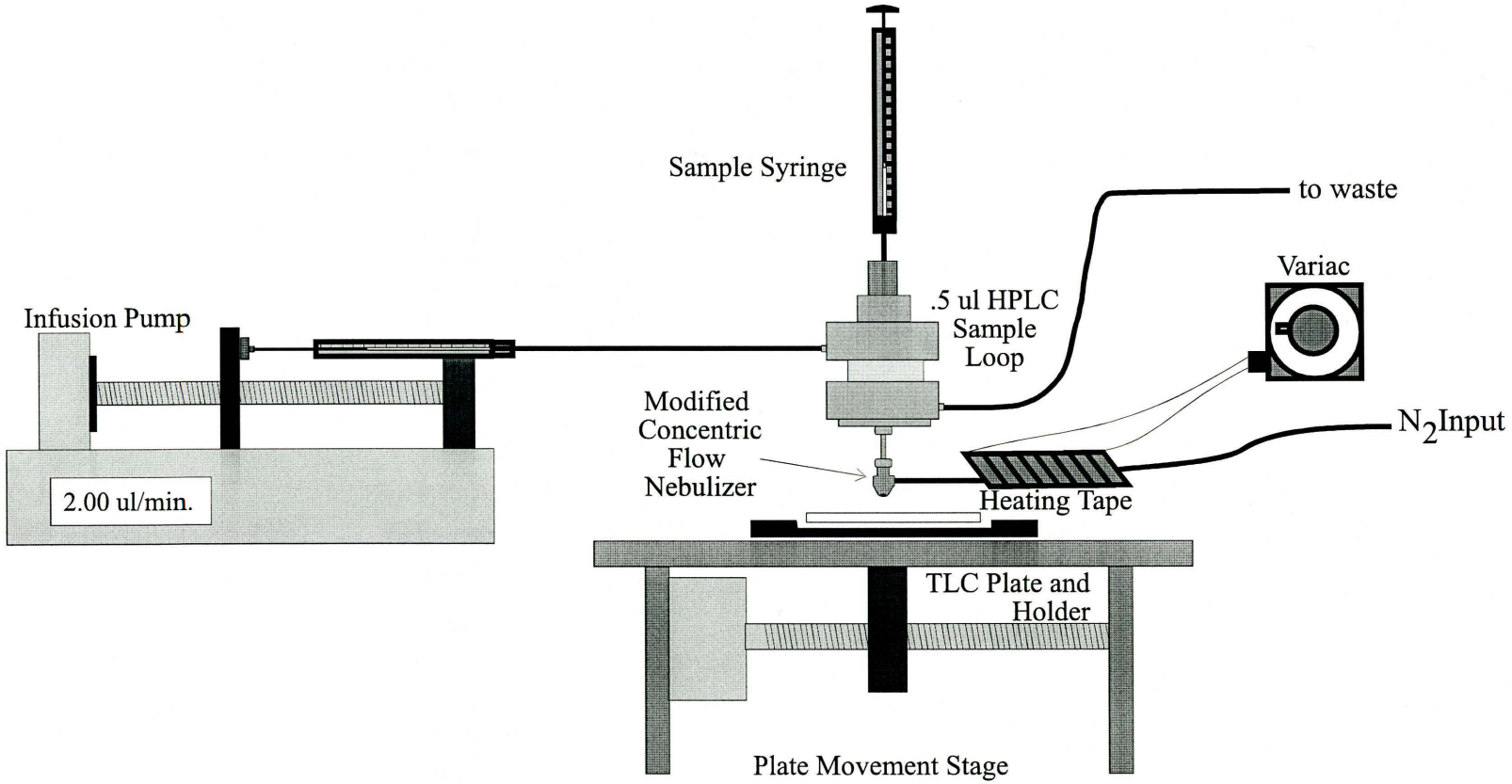


Figure 2.5, Modified sample applicator system
The system includes the modified nebulizer head, temperature control and automation capabilities

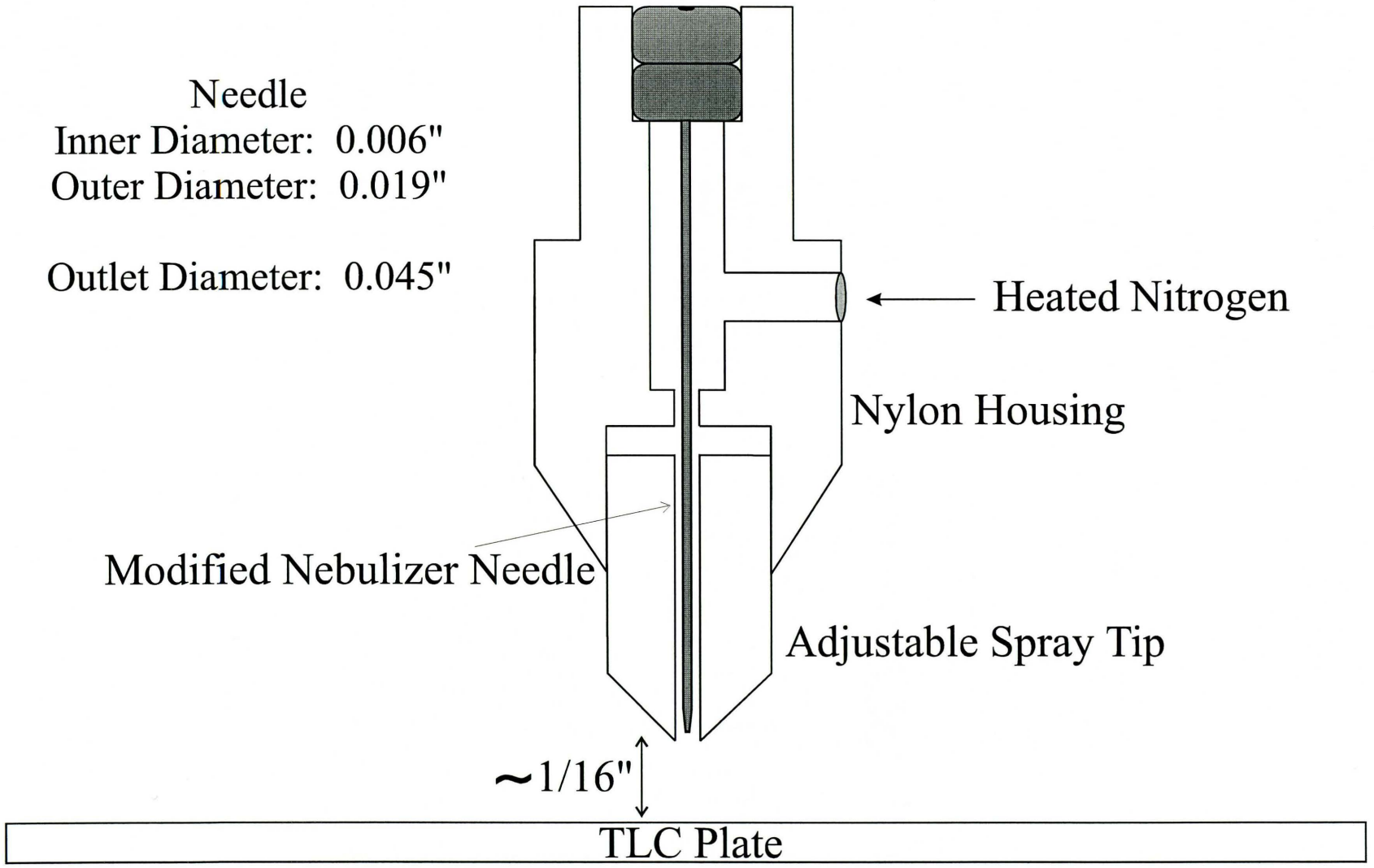


Figure 2.6, Modified Micro-Nebulizer Head

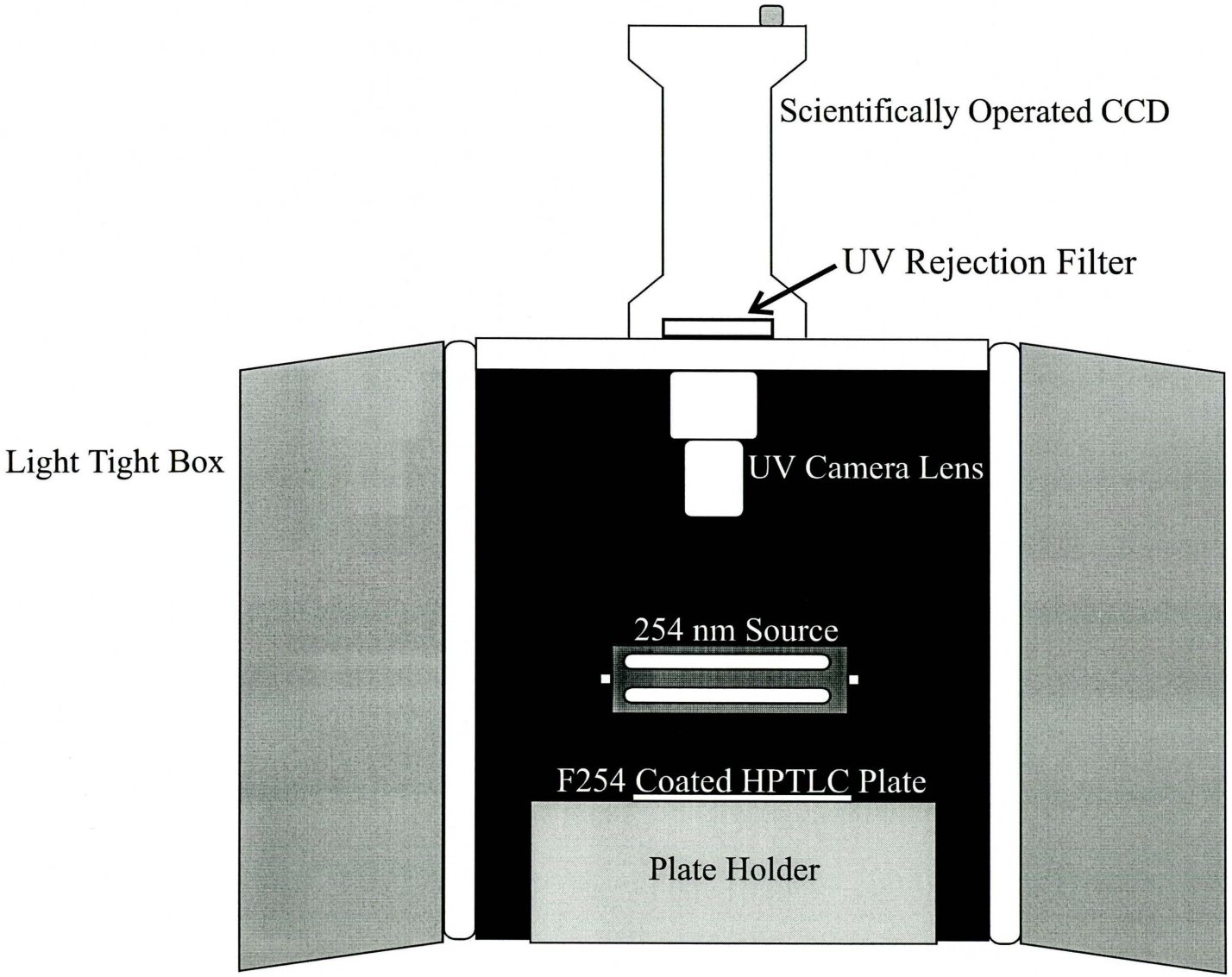


Figure 2.7, Modified illumination and detection arrangement

Results

System Precision

The precision of the system was evaluated by applying several sets of a series of 4 spots of FD&C blue dye #1 onto the fluorescently coated plates. The spots were integrated using a Labview based (Labview, National Instruments Corporation, Austin, TX.) program designed to integrate varying spot areas and give statistics on the integrated area. The results were as follows:

Trial	n	%RSD
1	4	1.21%
2	4	0.98%
3	4	0.97%
4	4	1.31%
5	4	0.71%
6	4	0.45%
7	4	0.70%
8	4	1.13%
9	4	0.85%
10	4	0.98%
	Pooled RSD	0.93%

Table 2.2: Modified System Precision

The results show substantial improvements over previous system design.

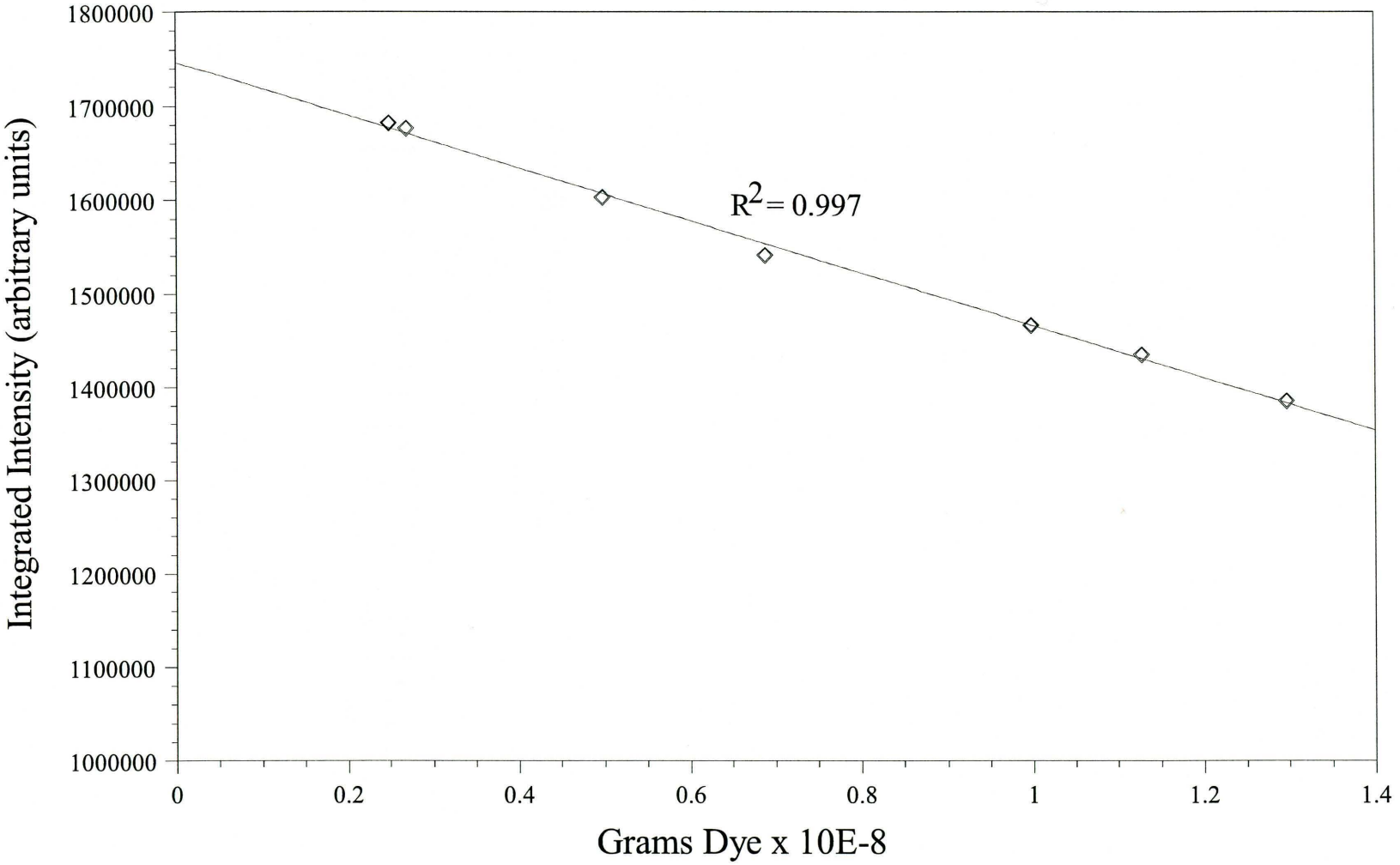


Figure 2.8, Accuracy of modified sample applicator

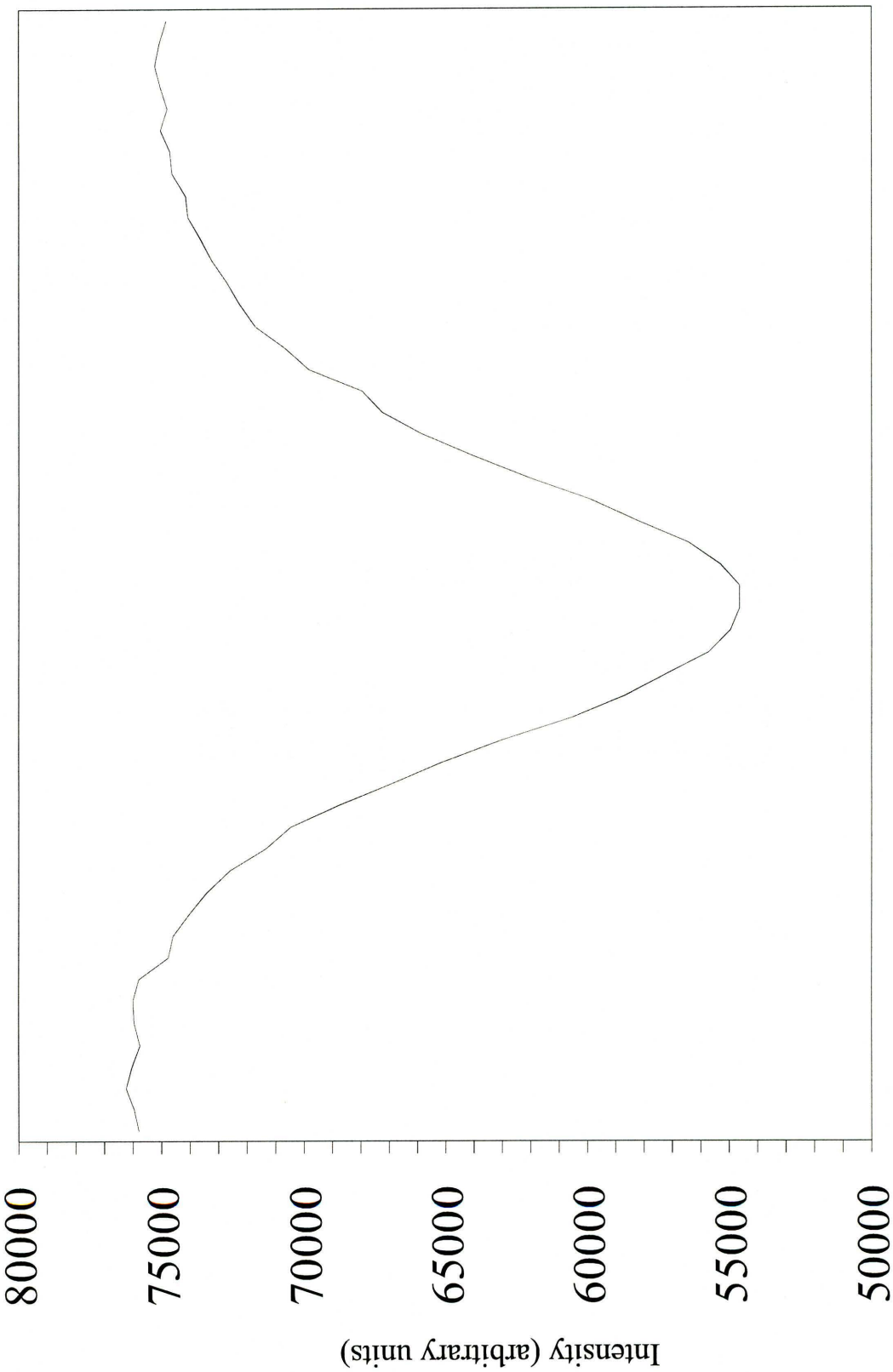


Figure 2.9, Spot profile of applied spot

Accuracy

The accuracy of the applicator was determined by applying varying concentrations of dye to the plate. The integrated intensity was plotted against the concentration of dye on the plate. Results were as shown in Figure 2.8.

Discussion

As shown, the system shows definite improvement in accuracy and precision over the previous design. The improvement in precision of the unit from 2.4% to 0.93% is a compilation of several variables. One of the problems associated with the original system was the solvent bubble that formed on the tip of the needle. This bubble was extremely prone to system shock and vibration. This occasionally caused the solvent bubble to dislodge and drip onto the HPTLC surface. When this happened, the spot chromatographed with the methanol as the spot spread out on the plate. This effectively ruins the spot, causing it to be several millimeters large and donut shaped. This is the reason that some of the precision results in the original spotter section have a population of 3 instead of 4. The new sharpened needle design does not have this problem since the solvent bubble does not form. The adjustability of the nebulizer head output also helped improve spot application and spot size. The ability to move the tip of the nebulizer head allows the best choice of spot focusing to be made, which is important in determining spot shape and size. The best configuration for the nebulizer head tip in relation to the needle tip was to place the needle approximately 3/4 mm inside the nebulizer tip. If the needle is flush with the tip or extends beyond the tip, spot focus is poor and the spray is unfocused and "spits" the analyte as it applies it to the plate. When the needle is slightly

inside the nebulizer tip, the gas flow seems to draw the liquid out just as it reaches the tip of the needle. This produces a very focused spray and spot size can be on the order of 1 mm or less with excellent precision. Another improvement in the system was the inclusion of the heat source on the nitrogen inlet. This allows for varying amounts of heat to be applied to the spray as it leaves the needle. This allows for the easier evaporation of less volatile solvents to the plate so the spots be applied as dry as possible while using a solvent that best solubilizes the analyte or analytes of interest. The introduction of heat also allows for higher solvent flow rates to be used without the analyte being applied too wet. Faster flow rates allow for more rapid spot application which is advantageous for the system. Yet another source of improvement is the use of the improved detection and flat-fielding system. The improvement in flat field correction is an important aspect in correcting for uneven illumination as well as spatial inhomogeneities occurring on the plate. The ability to correct for both spatial fluctuations in the source and in the plate greatly improves the results in both accuracy and precision. When the HPTLC plate is slightly inhomogeneous, as it nearly always is, correcting for only uneven source illumination is not adequate. These plate inhomogeneities consist of fluorescent impurities distributed unevenly on the plate, and variations in layer thickness, which vary the amount of photons transmitted to the detector.

A source of error arising from the use of this method of flat-fielding is residual application solvent. If the spot is slightly wet when the plate is imaged, the solvent can absorb a fraction of the UV light and cause the spot have a lower integrated intensity than the dry analyte. This can cause error in spotter precision, but the contribution seems to be small due to the evaporation of most the application solvent as it is applied. Another source of error is the spatial and temporal fluctuations from the illumination source. The

mercury pen lamp used for illumination has a tendency to drift in intensity as time progresses. These drifts cannot be accommodated for by flat fielding. The uneven source illumination that is corrected for by flat fielding cannot take into account the uneven drift that the source exhibits. If the source spatially drifted evenly across the plate, the correction would not be so difficult, but the source drifts unevenly and as a result, there is an inherent error from the illumination. For example, the upper left hand corner of the plate may drift by about 1% over the course of about 10 minutes while the upper right hand corner may drift as much as 1.5%. This temporal fluctuation cannot be accounted for and can introduce error into both precision and accuracy measurements.

Another source of error is from the precision error in the HPLC injector loop used to measure the sample before application. Rheodyne quotes the percentage of error in its injector at about 1%. This probably represents the fundamental precision limit the unit can exhibit. The results from the precision data shows a variance due mostly to source drift, and if this were completely eliminated, the precision of the unit would be at about 1%. Other sources of error, such as detector shot noise, and operator error in choosing integration area are minor contributors to overall system error.

The distribution of the analyte within the spot was analyzed and found to be very nearly Gaussian shaped. The spots were roughly 1 mm in diameter. Different solvents for nebulization were tried and no significant change was noted in either the size or shape of the spot. Chloroform, water, acetone, and toluene were all tried using the same concentration of dye solution and the spots were analyzed. The only difference noticed was the slight difference in perceived spot intensity between the three solvents. This was due to the fact that the three solvents differ in their ability to vaporize and under identical spotting conditions for all three, this caused them to go down in differing degrees of

wetness. This caused the dye to be distributed further into the silica, making the spots appear lighter or darker, depending on the degree of solvent evaporation

Future Directions

Although the system operates within the criteria provided at the start of this chapter, further modifications would make the unit more efficient. The first improvement that would help improve precision is the use of a more stable illumination source for detection. This would not help actual system precision, but it would help better detect the true level of precision and accuracy that currently exists, without introducing the further increase in error from the source drift. Another area that could be improved is the use of more uniform plates. Again, this does not improve the precision or accuracy of the unit, but again it helps determine the true precision and accuracy of the unit. The removal of localized fluorescent impurities, particularly in the fluorescent detection mode, would greatly improve the overall technique for determining system performance. Another way to increase system performance would be to introduce a more accurate way of positioning, and more importantly, repositioning the HPTLC plate for detection. Currently, flat fielding is not as good as it could be for eliminating the localized fluorescent impurities occurring on the plate. An repositioning error of 1 or 2 pixels can, if the impurity occurs at the same location as an analyte, limit the usefulness of the flat field correction and hurt quantitation of the effected analyte.

System flexibility could be increased by implementation of band application for both linear and circular development. This would allow for more flexible development procedures and increase the overall utility of the system. Another improvement would involve the utilization of simultaneous application of several spots. One of the slowest steps in the overall separation process is the application of the sample. This could be

remedied by the use of several nebulizer heads operating in parallel to apply several spots at once. The entire system could even be automated for operator-free operation. An additional improvement would be a reduction of the dead volume between the sample loop and the nebulizer head. This would have the effect of reducing the required application time for each individual spot. Finally, the utilization of smart software would greatly increase system operability. The implementation of software that could image the entire plate, automatically integrate the spot area of analytes on the plate, and inform the operator of relative spot intensity of every spot on the plate would greatly reduce analysis time. A simple criterion for determining whether or not a spot is present would be programmed into the system package, and the computer would quickly determine the probability of spot presence on the plate.

CHAPTER 3

QUANTITATIVE ANALYSIS OF AFLATOXINS BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY UTILIZING A SCIENTIFICALLY OPERATED CHARGE-COUPLED DEVICE DETECTOR

Background

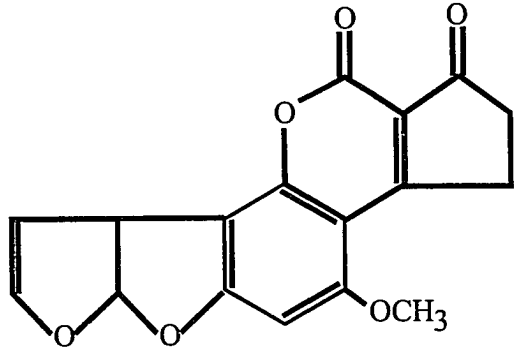
Aflatoxins are toxic, teratogenic, mutagenic, and extremely carcinogenic substances excreted by green, powdery molds known as *aspergillus flavus* and *aspergillus parisiticus*. Aflatoxins contaminate agricultural products worldwide, and a large number of deaths are caused each year by undetected aflatoxins in food products.(3.1) Aflatoxins are not only found in foods such as corn, peanuts, and rice, but are also found in animal products such as meat and dairy foods (3.2). This is due to aflatoxin contaminated feed being fed to animals which contaminates all products from that animal. Monitoring for the presence of aflatoxins is an important step in reducing cases of aflatoxicosis, poisoning by aflatoxins, as well as cancer caused by continual consumption of low to moderate amounts of aflatoxins. Strong correlation has been found between levels of aflatoxin in human diets and the incidents of liver cancer.(3.3) While improvements in storage conditions can greatly reduce the problems associated with aflatoxin contamination, extensive, routine monitoring for these compounds at every level of production, including growing, harvesting, storage, and processing, are required to control this severe health hazard.(3.4) A low cost and reliable technique for fast and efficient analysis of large amounts of potentially contaminated samples is necessary for practical, routine monitoring of these hazardous compounds. The technique requires a high degree of sensitivity for the aflatoxins under investigation, in order to prevent the

need to work with large sample sizes, thus minimizing technician exposure. An additional requirement for the monitoring of aflatoxins is the need for very thorough sampling. The problem with sampling is associated with the fact that there is not a normal distribution of aflatoxins within one batch.(3.5) Thus a large number of samples must be taken to ensure that a good representation has been acquired. This requires that the separation and detection system have sufficient throughput to analyze all of the samples in as short a time as possible.

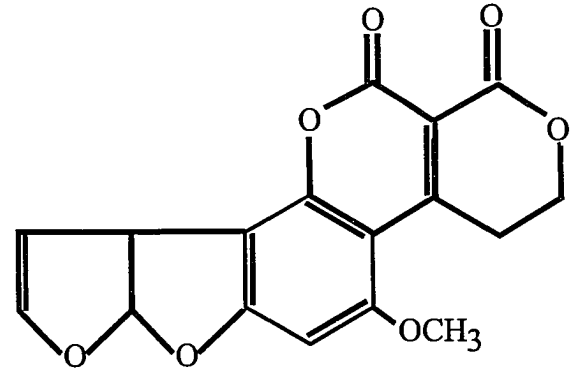
Two techniques have been used extensively for the analysis of these substances, high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) with fluorescence detection.(3.6) Unfortunately, these techniques, as employed in the past, are an impractical means of rapid, routine, quantitative analysis. HPLC, while providing for a good means of quantitative analysis for aflatoxins, requires that all standards and unknowns be injected sequentially onto the chromatographic column. Sequential analysis in this manner takes place at the expense of time, solvent consumption, and waste disposal. This makes HPLC a less than ideal choice for the quantitation of aflatoxins.

HPTLC has all the requirements necessary for the separation of aflatoxins, but the mode of detection needs to be significantly improved in order to compete with the column techniques that are currently more popular. The limiting step in an aflatoxin separation performed on a HPTLC medium is detection. The method of detection needs to be greatly improved in order to take full advantage of the high throughput of HPTLC. The current method for quantitation of analytes on the HPTLC plate is scanning densitometry. The scanning densitometer operates by rastering the separation lanes with a light beam in the form of an adjustable slit. The difference between the optical signal from the sample-free background and the signal from the analyte spots corresponds to the

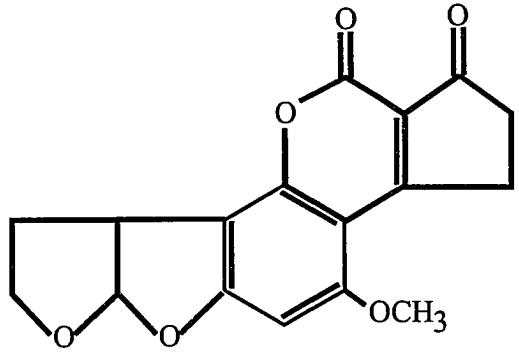
amount of analyte on the plate. The instrument can employ single or double-beam geometry, and can be run in reflectance or transmittance mode using absorbance or fluorescence detection. This is a sequential technique, meaning the instrument scans a plate lane by lane, and the spots are detected one at a time. While providing good quantitative results, the technique is slow and reduces the throughput of HPTLC, its main advantage, to the level of linear techniques, since each spot must be individually read out. Despite drawbacks, the scanning densitometer is currently the most popular means of quantitation in HPTLC. However, recent advances in Charge-Coupled Device (CCD) technology have made significant progress in the areas of detection and quantitative analysis for HPTLC.(3.7)



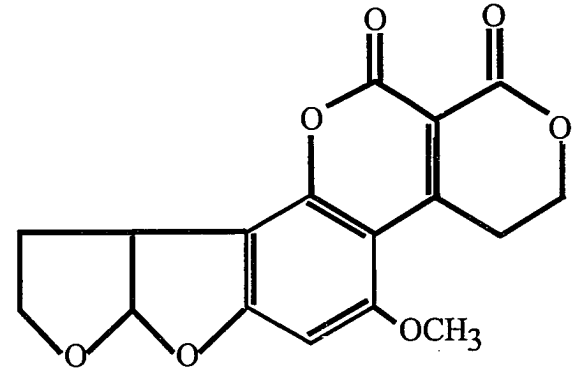
B1



G1



B2



G2

Figure 3.1, Structures of the aflatoxins under investigation

Charge-Coupled Devices

Charge coupled devices or CCD's are two dimensional imaging arrays that are perfectly suited to the planar format of the HPTLC medium. Rather than scanning the plate one lane at a time, the entire plate is imaged and analytical information can be rapidly extracted from the image. CCD's are described in the following section:

Scientifically operated CCD's are extremely sensitive detectors that detect wavelengths extending from the deep UV to the Near-Infrared regions of the spectrum. Some of the characteristics of these detectors are:

- Wide Wavelength Coverage
  - deep UV to near-IR
- Large Linear Dynamic Range
  - > 4 orders of intra image dynamic range
- High Quantum Efficiency
  - peak QEs exceeding 90% in the visible wavelength region
- Photometrically Accurate and Precise
  - linear over entire dynamic range
- High Spatial Resolution
  - > 4 million detector elements with a 24 μm square pixel size
- Extremely Low Noise Operation
  - ~ 4 electrons at 40 kHz readout rates
- Extremely Low Dark Current
  - less than .01 electrons/pixel/second @ -100 ^{o}C
- Robust
  - no damage when exposed to bright illumination

CCD's were first conceived of as an electronic analog of the magnetic bubble device. Today, the CCD is primarily used as an imaging tool. In the past two decades, the main goal of the CCD manufacturer was to develop a detector that would replace the tube type sensors used at the time. Today, the CCD is rapidly replacing tube based detectors and photographic film as the detector of choice in many imaging applications.

Charge Generation and Collection

To function as a detector, the CCD must perform four primary tasks: 1) charge generation, 2) charge collection, 3) charge transfer, and 4) charge detection. The first of these tasks, charge generation, relies on a phenomenon known as the photoelectric effect. This occurs when a photon strikes certain materials and free electrons are generated. Silicon exhibits a bandgap of approximately 1.14 eV situated between the valence and conduction bands. When a photon of sufficient energy strikes the silicon, valence electrons are transferred to the conduction band and an electron-hole pair is created. The electron-hole pairs are free to move about in the silicon lattice structure until they recombine, typically 100 microseconds after creation. The useful photoelectric range for silicon extends from the near infrared region (1.1 eV) to the soft X-ray region (10 keV) of the spectrum. Photons lower in energy than 1.1 eV do not have sufficient energy to elevate an electron from the valence band to the conduction band. Photons with energies greater than 10 keV have such a small interaction probability that the silicon is transparent to these wavelengths.

Once the charge has been generated, it needs to be collected. In a CCD, the fundamental building block is the Metal Insulator Semiconductor (MIS) capacitor. The MIS capacitor is fabricated on p-type (boron doped) epitaxial silicon on which an insulator layer of silicon dioxide and silicon nitride is grown. This layer is followed by a conductive gate deposition, typically doped poly-silicon. When a positive voltage is applied to the gate, a depletion region of the majority carrier (holes) forms. The potential variations within the depleted silicon causes a potential well for electrons at the surface to form. The CCD is composed of closely spaced MIS capacitors. The simplest configuration is what is known as a 3-phase device. In a 3-phase device, a number of gates are arranged in parallel with every third gate being connected to the same clock

driver. In the CCD, one picture element, or pixel, consists of a triplet of these gates, each connected to phase 1, 2, and 3 clocks. To collect the charge generated by a photon interaction, one of the gates is biased high, around +10 Volts, forming a depletion region relative to the other 2 gates which are biased at +5 Volts, for example. When an electron-hole pair is generated, the electrons can now be collected under the gate that is biased high, known as the collecting phase. The other two gates are acting as the barrier phases, preventing collected electrons from spilling into adjacent pixels. A CCD array can be thought of as many shift registers composed of many pixel elements. The active region of the chip is covered with closely spaced vertical registers or columns, separated by implanted channel stops to prevent charge spillage between columns.

Charge Transfer and Readout

Once the charge has been generated, it must be transferred to a region of the chip where it can be read out. In a CCD, this is accomplished by manipulating the voltages on the gates in such a way that causes the generated charge packets to shift down the column to a location where they can be read out. At the end of the columns is a horizontal register of pixels. This register collects the charge packets one line at a time and then transports the charge packets in a serial manner to an on-chip amplifier. The final step in the process involves converting the individual charge packets into voltages, which can be amplified off chip, digitally encoded, and manipulated in the appropriate manner.

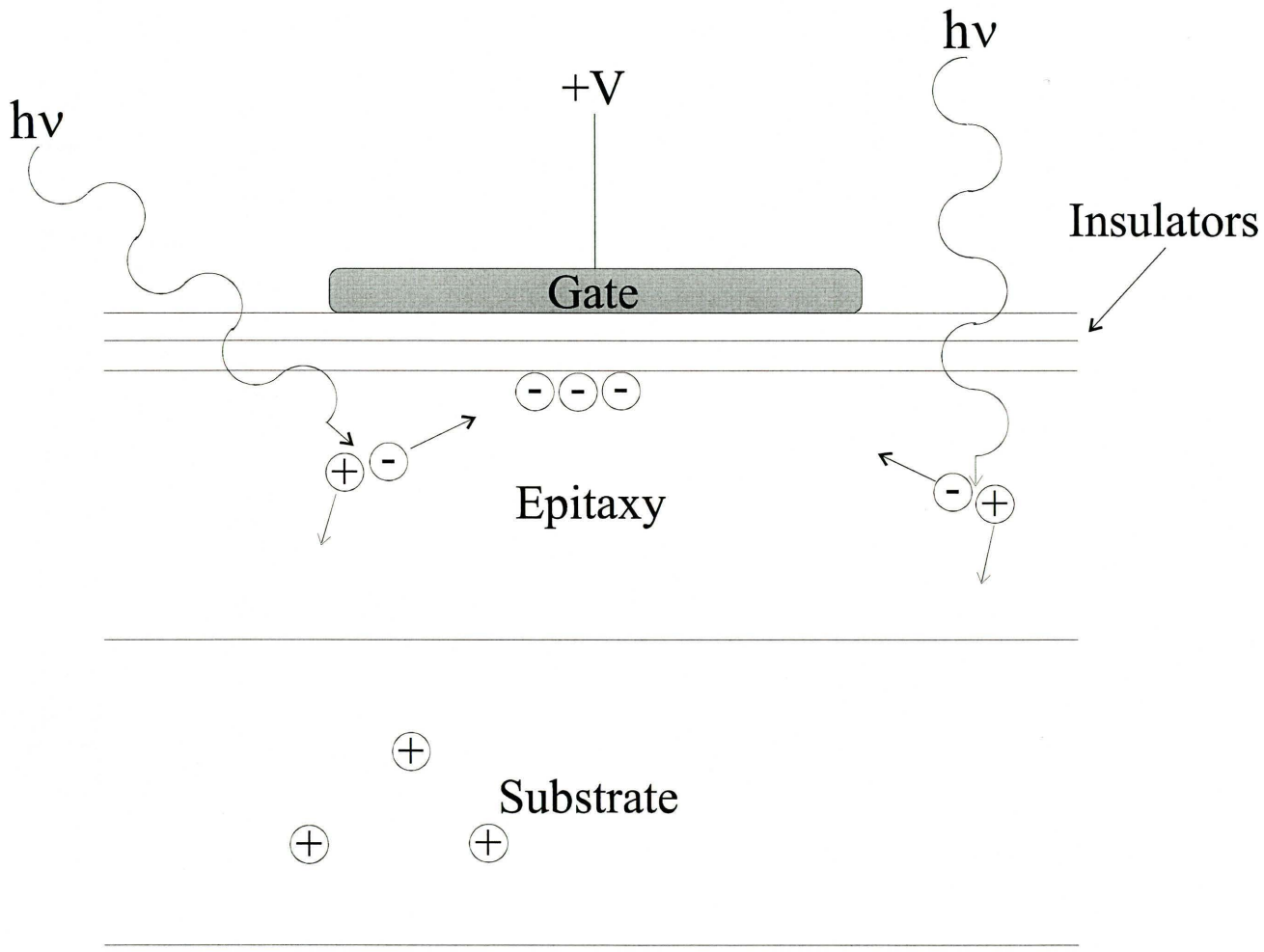


Figure 3.2, Charge generation in a CCD

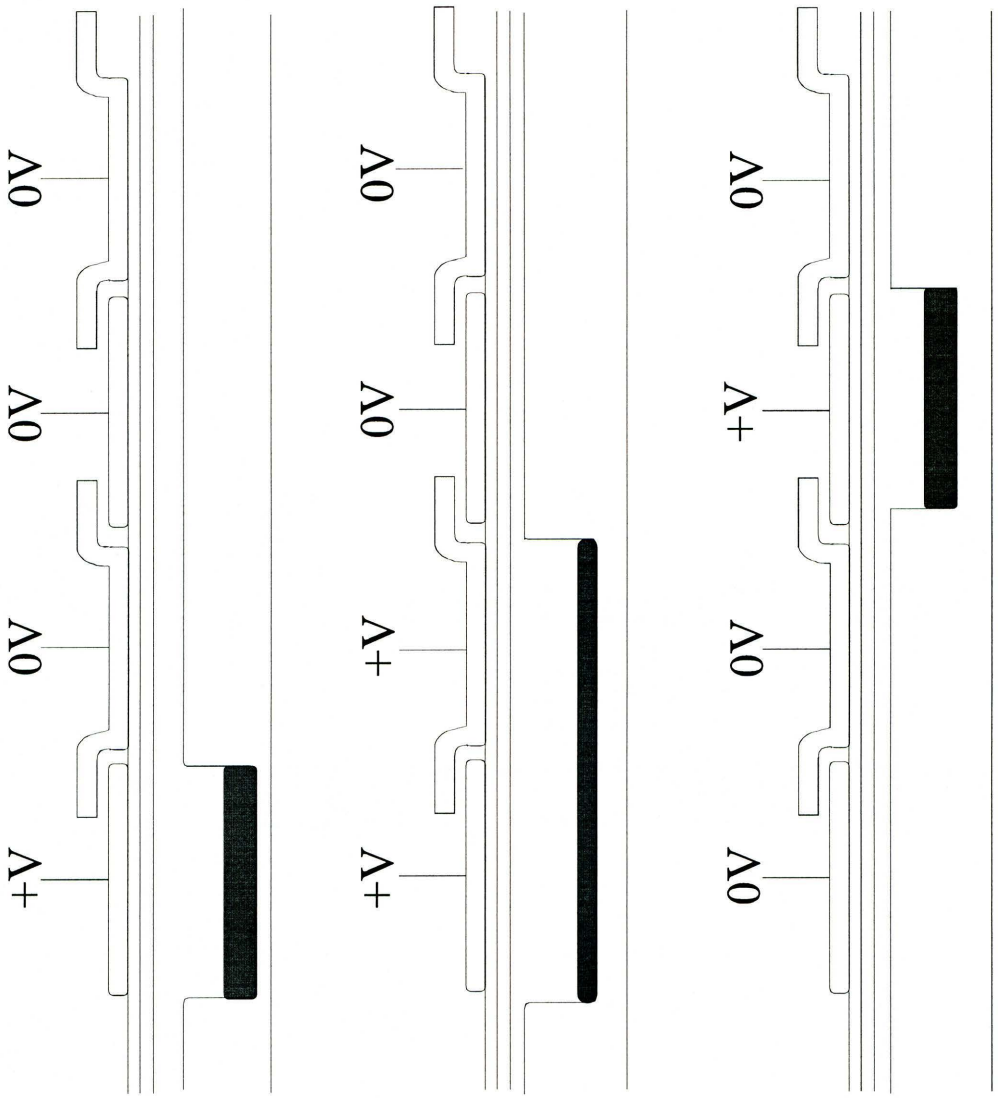


Figure 3.3, Charge transfer in a CCD

Buried Channel CCD's

The CCD's described previously are what is known as surface channel CCD's. This means that the charge is collected, stored, and shifted along the surface of the semiconductor, at the Si-SiO_2 interface. A considerable problem exists with this type of operation, in that charge can become trapped in what are known as interface traps, and this severely degrades the efficiency of charge transfer performance (3.8). It became clear that surface channel devices were not suitable for scientific applications, where charge transfer efficiency is critical. A solution to this problem involved development of what is known as buried channel devices. In a buried channel device, charge transfer along the surface interface is avoided by implanting a channel of n-type material that lies below the interface. This causes electrons to collect below the Si-SiO_2 interface and circumvents interface traps, raising charge transfer efficiency to levels suitable for scientific work.

Backside Illumination

To achieve a broader spectral response region, improvements were needed in the CCD. In the early development of the CCD, the spectral response of the detector only covered the Near-IR, the visible, and some of the X-ray region. This was due to the fact that the chip was illuminated on the front side, meaning the photons had to pass through the relatively thick gates before they could be detected. Photon absorption in the gates was the cause of the relatively poor response of the detector in certain regions of the spectrum. A solution to this problem involved a process known as backside thinning. In this process, following wafer fabrication and die separation, the backside of the CCD is thinned and the newly exposed surface treated to ensure a high quantum efficiency. An additional advantage of the use of backside illuminated architecture is the ability to use an anti-reflection coating on the chip. This can reduce reflective loss of incident photons at

the highly reflective silicon surface to nearly zero at the design wavelength. The chip can now be illuminated from the backside, avoiding the absorption of photons by the gates, and the spectral response range and quantum efficiencies of the newly thinned chip are greatly improved upon.

Scientific Operation

In order for the CCD to exhibit the extremely low noise required for most scientific applications, it must be operated in what is known as scientific operation mode. Most CCD's used today are operated in what is known as video mode. Video mode requires a fast readout rate, usually 30 frames/sec, with little regard to noise performance or low light level performance. To accomplish this rapid readout, half of the photosensitive region is masked and used as a transfer region to transfer photogenerated charge for readout while the next frame is integrating. This is performed very rapidly and without the use of a shutter, typically with 8-bits of digital precision. This mode of readout is unsuited for use in many scientific operations, which require lower noise and higher digital precision, typically 14 to 16-bits. Conversely, scientific operation of the CCD requires a lower level of noise and higher spatial accuracy and precision to be used in most scientific applications.

To achieve the performance necessary for most scientific applications, the CCD must be cryogenically cooled to reduce the buildup of thermally generated charge. If operated at room temperature, thermally generated charge can rapidly accumulate at the rate of thousands of electrons per pixel per second. The full well capacity of the pixel would quickly be reached and integration times available to perform low light level imaging would not be possible. However when cryogenically cooled, typically to -100

°C, dark currents can be reduced to as low as .01 electrons per pixel per second, allowing for integration times on the order of hours if necessary.

Scientifically operated CCD's employ the entire photoactive area of the chip, since fast readout rates are usually not required. This requires the use of a shutter to mask the chip when it is not being used or when readout is performed. This doubles the photoactive area of the chip available to obtain scientific data. The quality of the chips is also higher, with multiple inspection stages during the manufacture process, to ensure the device is free of such defects as shorted pixel rows, cosmetic defects, or abnormally high regions of localized dark current.

Another difference between video mode and scientific mode of readout is the use of specialized readout electronics. Charge sensing in a CCD is performed as the electrons are shifted from the serial register to a MOS gate, where the voltage change is measured. The voltage change is proportional to the amount of charge within the packet. The major source of noise from this type of readout comes from switch noise in the output amplifier circuitry, typically in the hundreds of electrons. To eliminate this source of noise, the scientifically operated CCD employs a technique known as double-correlated sampling. In this mode of readout, the charge is readout before and after it has been dumped to the output. When measured in this manner, the change in potential is independent of the switch noise. This readout mode, along with slower readout speeds and specialized electronics reduces the read noise typically to as low as 4 electrons at 40 kHz.

Experimental

Aflatoxin Standards

Aflatoxin standards B1, B2, G1 and G2 utilized for system evaluation were obtained from Sigma Chemical Co. (Sigma, St. Louis, MO, cat. no. AF-1). The aflatoxins were received in crystalline form. Each of the 1 mg standards were dissolved in 2.0 ml of analytical reagent grade methanol. Each of these four standard solutions constituted the stock solutions from which all further quantitative standards were prepared. All standards were made by further dilution of the stock solution with reagent grade methanol.

Sample Application

The original sample applicator described in Chapter 2 was used to apply analytes to the plate. The flow rate for the solvent delivery system was 4.00 μl/min. and the nitrogen nebulization pressure was 27 psi on the regulator. The spots were deposited for 6 minutes. Spots were approximately 3 mm in diameter.

HPTLC Separation

For linearity and precision measurements in this study, aflatoxins were separated by vertical development in a beaker. Macherey-Nagel brand silica gel HPTLC plates (Macherey-Nagel Nano-Sil 10) were utilized for the separation. All plates were develop washed with MeOH for two minutes, and allowed to dry prior to separation in order to reduce the fluorescence background from any adsorbed organic impurities. Separation of the aflatoxins for real time, in-situ visualization was carried out in a horizontal HPTLC developing chamber (Sargent-Welch, Part # S18930-01). The mobile phase consisted of 90/10 (v/v) chloroform/acetone. The chamber was modified in such a manner so as to allow for the passage of UV light through the bottom of the chamber. This was

accomplished by cutting a 1.5" by 1.5" hole in the bottom of the chamber and support plate and replacing the cut out section of the chamber with a 1/16" ground and polished fused-silica window (GM Associates Inc., OZ Grade). The fused silica plate was utilized to seal the chamber so as to allow for a two minute water vapor pre-treatment of the chromatographic surface and the passage of UV light for fluorescence excitation.

Illumination

The aflatoxins were detected by illumination by a specially constructed transilluminator system. A 15 cm by 15 cm hole was cut in the top of an aluminum box (10" by 4" by 17"). Over the hole was mounted a wide bandpass UV filter (Hoya Optics, Fremont, CA, U-340, 3.0 mm by 165 mm by 165 mm). Two 8 watt, long wavelength UV (365 nm) fluorescent light tubes (Spectronics Corp., Westbury, NY, BLE 8T365) were mounted in the box. Illumination of the plates was achieved by placing the silica surface of the chromatographic plates in direct contact with the UV pass filter (glass side up). For the real-time separation, the plate was placed silica side down and illuminated through the modified horizontal chromatographic chamber.

Imaging System

The imaging camera system utilized in this study was provided by Photometrics, Ltd. (Tucson, AZ). The system consisted of a CC200 camera controller, CE200 camera electronics unit and a PM512 scientific grade CCD. The CCD was mounted in an evacuated dewar assembly, which allowed for cryogenic cooling of the device to -100°C to prevent accumulation of dark current. The exposure time was controlled with an electronic shutter mounted between the dewar assembly and lens. The shutter mount also allowed for the adaptation of a f4.5, 105 mm focal length UV Nikor lens (Nikon) which

acted as the imaging optics. A CVI Laser filter (CVI Laser Corporation, LWPS-400, Albuquerque, NM.) was mounted between the shutter and camera faceplate to achieve the proper emission filtering characteristics for the aflatoxins under investigation. The system configuration is shown in Figure 3.4. All images were bias subtracted to remove electronic offsets and flat fielded to correct for variances in source intensity. Both bias subtraction and flat fielding are described in Appendix A. Exposure times were 300 seconds.

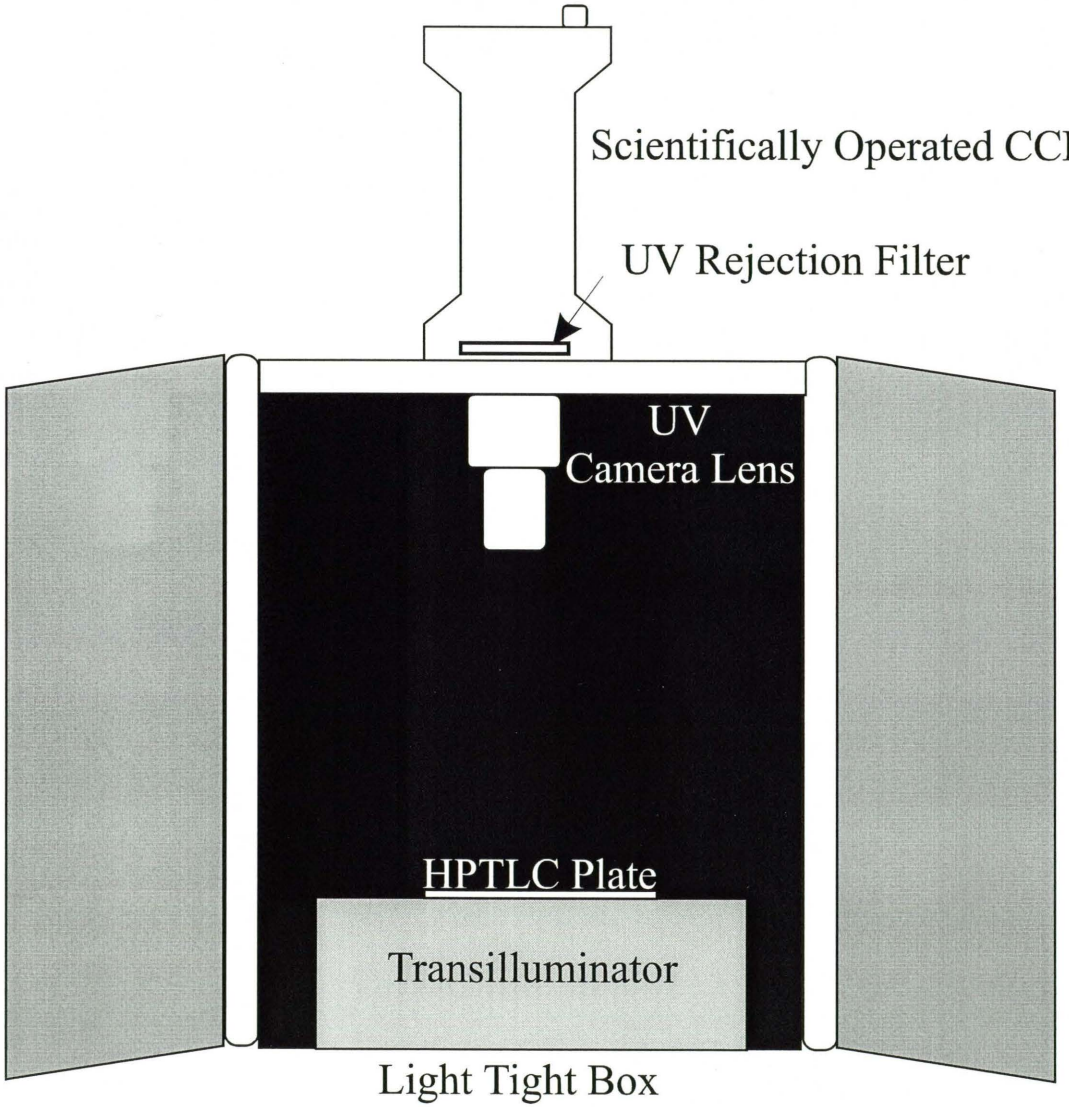


Figure 3.4, Aflatoxin detection arrangement

Results and Discussion

Precision

System precision was evaluated by applying replicate 515 picogram samples of aflatoxin G1 onto the chromatographic plate. The fluorescence of each of the spots was integrated using the CCD before and after chromatography. All images were bias and flat field corrected. The results of the inter spot precision are shown in Table 3.1. Inter-image precision was evaluated by taking ten successive exposures of the fluorescent flat field filter and measuring the mean intensity of two 32 by 32 pixel subarrays located near the center of each image. The arrays were separated vertically by 100 pixels. The mean signal intensity for the two pixel array boxes and the standard deviation of the difference in mean signal intensity between the two arrays was determined for all ten trials. The %RSD for inter-image precision in the system was found to be less than 1%.

Trial	# of Samples	%RSD before separation	%RSD after separation
1	3	3.9	11.8
2	3	2.0	9.4
3	3	1.5	7.3
4	4	1.4	4.0
5	4	.8	7.0
6	4	2.7	7.1
7	4	4.0	5.6
8	4	3.5	3.7
9	4	1.5	3.9
	Pooled %RSD	2.4	6.4

Table 3.1, Interspot precision results.

The inter spot precision represents the combination of several variables and sources of error. The variation in source intensity over time contributes to the amount of excitation energy reaching the aflatoxins, and consequently, their fluorescent intensity. Plate inhomogeneities in the form of fluorescent impurities are also major contributors to system imprecision. There are two types of fluorescent impurities that are present on the plate. The first type of impurity is the background fluorescence present over the entire plate. This impurity can be removed somewhat, but not entirely by plate washing techniques. The second type of impurity is the localized fluorescent spots that occur

randomly on the plate. Most of these spots could not be removed by washing, and thus when these spots occurred in the same location as the aflatoxin spots, they introduced error into the measurement by adding the signal of the impurity to the signal from the aflatoxin. An additional source of error arises from the use of a separate flat field filter to correct for uneven illumination. While doing a good job at correcting for spatial source fluctuations, it can not correct for plate inhomogeneities such as silica thickness and localized fluorescent spots. The error in scientific CCD detection is considered negligible, being dominated over most of the working dynamic range by shot noise. This is true because of the low noise performance of the scientific CCD, the high background signal levels and the relatively high degree of fluorescent impurities in the chromatographic layer. Additional errors are introduced by the operators decision on the choice of peak position. This error is compounded by the presence of the fluorescent plate impurities if they occur near the analyte.

The results of the inter spot precision data shows that the chromatographic process itself is the largest contributor to imprecision, with the largest errors encountered after chromatography. This is due mostly to the low quality of the plate, particularly in the areas of particle size and particle size distribution. These imperfections cause the analyte to be distributed unevenly and inconsistently throughout the stationary phase after development. This can cause the analyte not to encounter the same UV illumination intensity as other analytes, being essentially "hidden" from source illumination. This alters the readings obtained for intensity and decreases system precision after the development process.

Dynamic Range and Sensitivity:

Detection limits (S/N = 3) were determined for each of the aflatoxins under investigation, as shown in Table 3.2. Excellent linearity was achieved over greater than an order of magnitude for all aflatoxins under investigation as shown in Figure 3.6.

Aflatoxin	Detection Limit
G2	1 Picogram
G1	2 Picograms
B2	3 Picograms
B1	3 Picograms

Table 3.2, Aflatoxin detection limits.

Image Detection and Enhancement

An important ability of the CCD for the attainment of analytical information of the aflatoxins is its ability to bracket analytical information on the display screen. The computer is able to scale the appropriate analytical information by adjusting the 256 level gray-scale display so that only aflatoxin fluorescence is shown, and lower concentrations of extraneous impurities are left out of the display. This ability of the system to show only pertinent analytical information is an important tool for the examiner. By showing only the fluorescence from the aflatoxins, the system operator can better choose the optimum location to integrate and quantitate the aflatoxins.

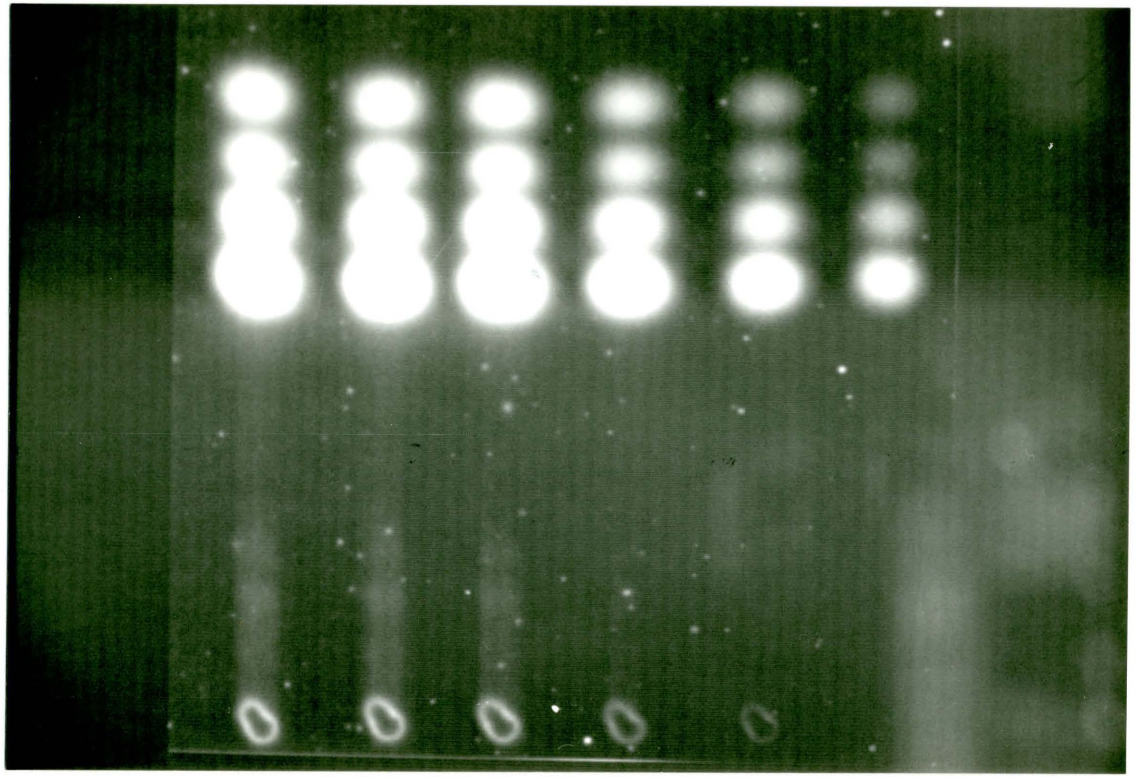


Figure 3.5, Image of varying concentrations of the separated aflatoxins

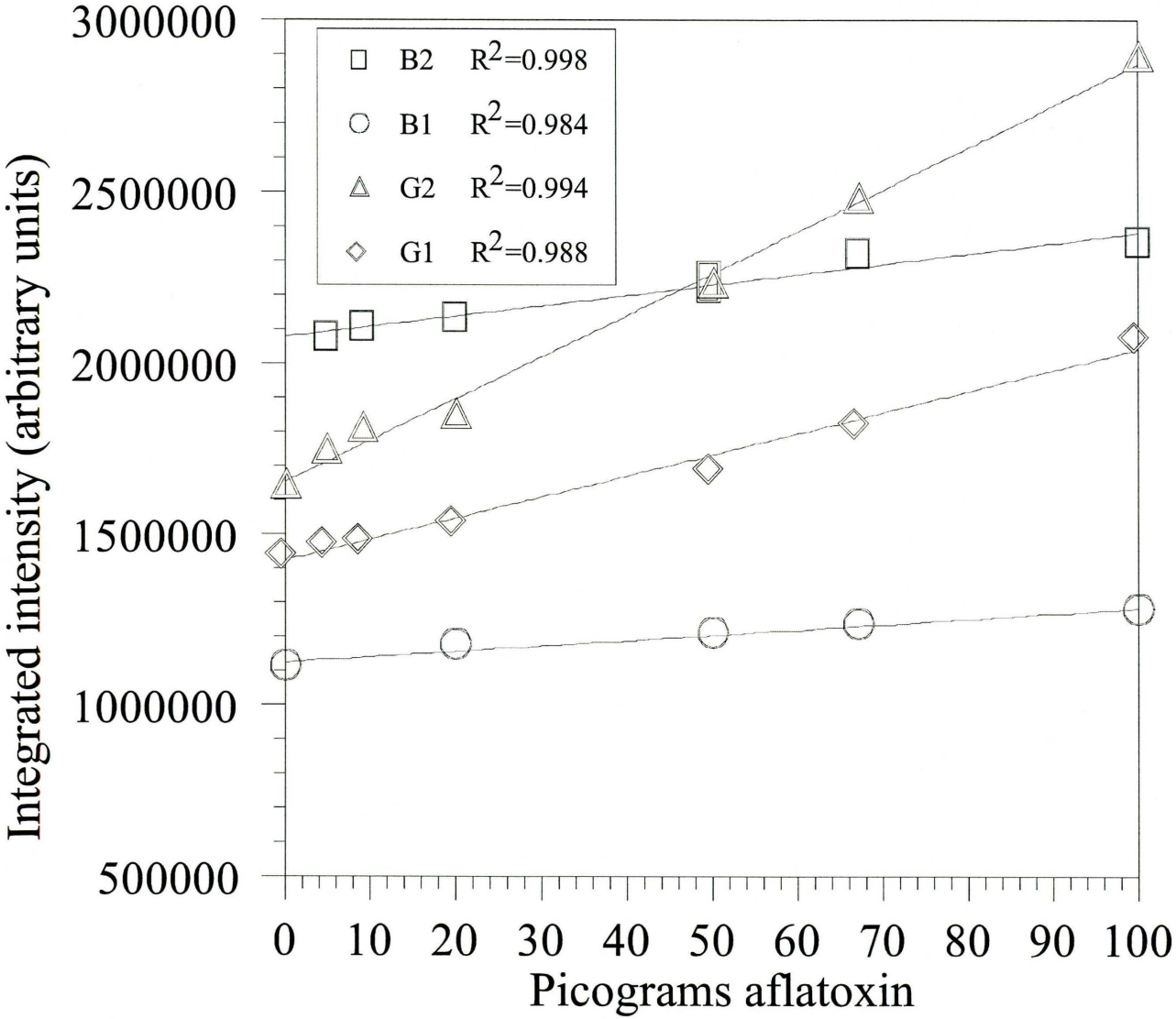


Figure 3.6, Aflatoxin calibration curves

Real Time, In Situ Monitoring

In HPTLC, the ability to monitor a separation as it progresses can be an important tool in determining to what extent a separation is complete. Plates can differ in their surface activity and homogeneity, which can alter their separation ability(3.9). Monitoring the separation as it progresses can signal the end of an adequate separation, avoiding over or under-developing the plate. Another advantage is that, if the separation is not proceeding as expected, the development can be stopped, reducing time wasted waiting for an inadequate separation to complete. In order to monitor the aflatoxins as they develop, a specially modified horizontal developing chamber was constructed. A hole was cut in the bottom of a Sargent-Welch horizontal developing chamber in order to utilize the transilluminator as the illumination source. The monitoring of the separation of aflatoxins B2 and G2 are shown in Figure 3.7. The progress was monitored at various times during the separation and it can be seen that the two aflatoxins are well resolved in 3 minutes 23 seconds. This preliminary work shows that real-time monitoring is possible to achieve optimal separation times of the aflatoxins under investigation.

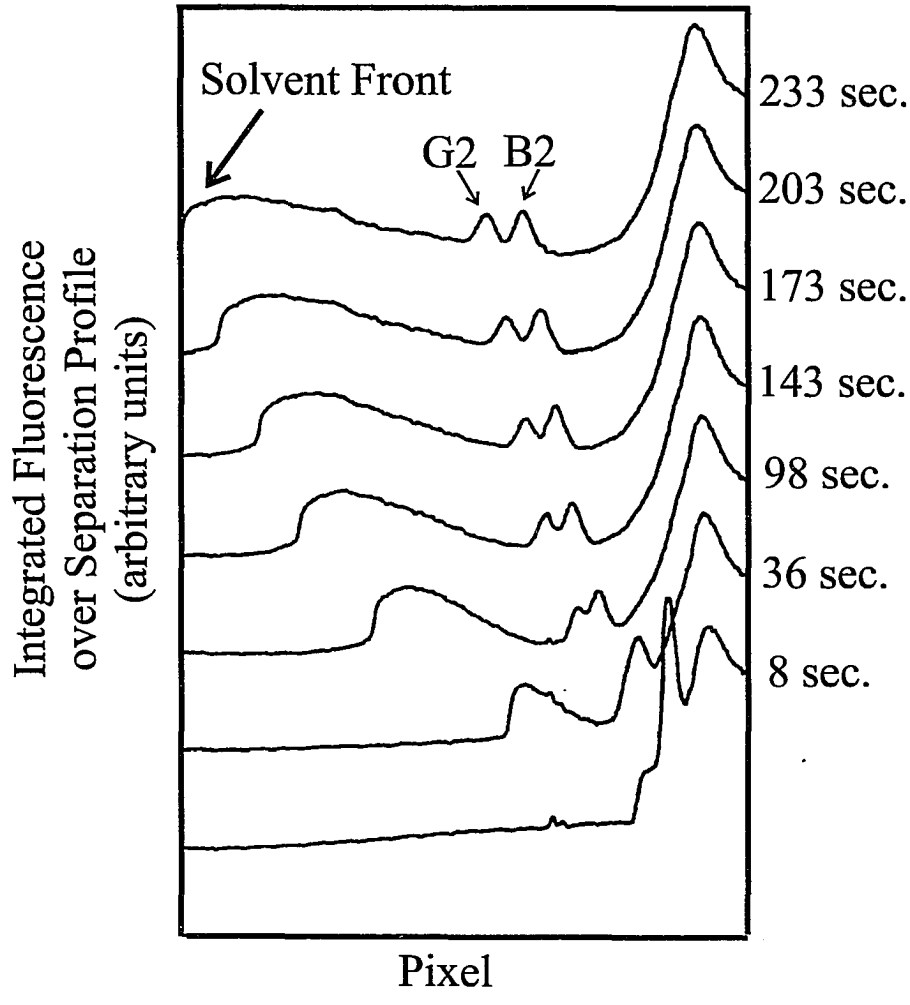


Figure 3.7, Real Time separation profiles of aflatoxins B2 and G2 at varying times during separation

Conclusions

The technique of using a CCD as a detector for the analysis of aflatoxins shows great promise. The excellent sensitivities and linearity over more than an order of magnitude, indicate that this technique could be applicable to other systems utilizing the separation of fluorescing compounds. Fluorescent impurities unremovable by plate pre-wash were the main limitation of the system in regard to its detection limits, but further improvements in plate design will eliminate this problem to a large extent, putting detection limits into the femtogram range.

The CCD has many advantages over conventional detector technology when imaging the HPTLC plate. The array format of the CCD is ideally suited to the planar format of the separation media and analysis times for analyte detection and analysis are greatly reduced. At the present time, the cost of the CCD imaging system is comparable to other methods of detection and analysis, such as scanning densitometers, and system costs of CCD's are constantly decreasing as the technology matures. This in addition to the time saved in the operation of the CCD, coupled with the increased throughput that accompanies its use brings the cost of the CCD system well below that of scanning densitometers. These advantages, when coupled with the ease of use of the CCD over the scanning densitometer, makes the CCD an ideal choice for the detection and quantification of aflatoxins in a HPTLC medium.

CHAPTER 4

USE OF A SCIENTIFICALLY-OPERATED CHARGE-COUPLED DEVICE TO IMAGE DANSYLATED AMINO ACIDS ON HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC PLATES

Background

Dietary protein supply is one of the major factors influencing the productivity of farm animals.(4.1) When one considers the food requirements necessary to sustain the vast human population today and in the future, one understands the importance of a high yield return of our farm animals, as well as maximizing the food invested vs. food returned. With a growing human population and decreasing land resources, maximum output of farm animals becomes critical and farm animal nutrition becomes equally critical. The past 2 or 3 decades have seen significant advancements in our understanding of amino acid nutrition and requirements in farm animals. Supplementation of the diet of animals with amino acids to enhance the quality of dietary protein has become common practice.(4.2) Figure 4.1 shows the effect of amino acid imbalance on the growth profile of rats, as determined by Harper and Rogers. (4.3) It can be seen that an improper nutritional balance in an animal can significantly effect the growth of that animal. These advances show that quantitative analysis or "fingerprinting" of amino acids in feed stuff is required for evaluating quality, stability, and occasionally provenience of the product.(4.4) Determination of free amino acids in potatoes, for example, is required when by-products of potato processing plants are used as animal feeds, for which the free amino acid content is regulated.(4.5) In the past, several methods have been used to determine amino acid content of food products. High performance liquid

chromatography (HPLC) is a common means for detecting amino acids and delivers excellent quantitative information.(4.6) Unfortunately, HPLC is a linear technique and system throughput is not to the desired level. This requires either that the sampling level performed is reduced to accommodate the low throughput of HPLC, or many separate HPLC separation units must be purchased at considerable cost, to perform at the throughput level desired. Additionally, sample cleanup can be extensive to avoid contamination of the column by non-migrating species. High performance thin layer chromatography (HPTLC) has been used in the past with promising results. However, the throughput level of the system was reduced to that of linear techniques by the use of scanning densitometry as a quantitation technique. This effectively eliminates one of the main advantages of using HPTLC as the separation technique. The rate of scanning densitometry also precludes the use of many useful product derivitizations, such as dansylation, to aid in the detection and sensitivity of the results. Scanning densitometry is not an option due to the fact that product lifetime of the derivatized product is often short, and analysis must be performed relatively quickly after the product is out of solution to avoid decomposition of the product. Scanning densitometry is often too slow to perform analysis of the derivatized product and this eliminates it as a viable quantitation technique. Ideally, a technique would allow the use of HPTLC as a separation technique by performing quantitative analysis rapidly, allowing the use of derivitization techniques such as dansylation to enhance detection and sensitivity of the products. This system would also give good quantitative results and have large sample throughput. This work describes the separation and quantitation of dansylated amino acids by high performance thin layer chromatography with CCD array detection.

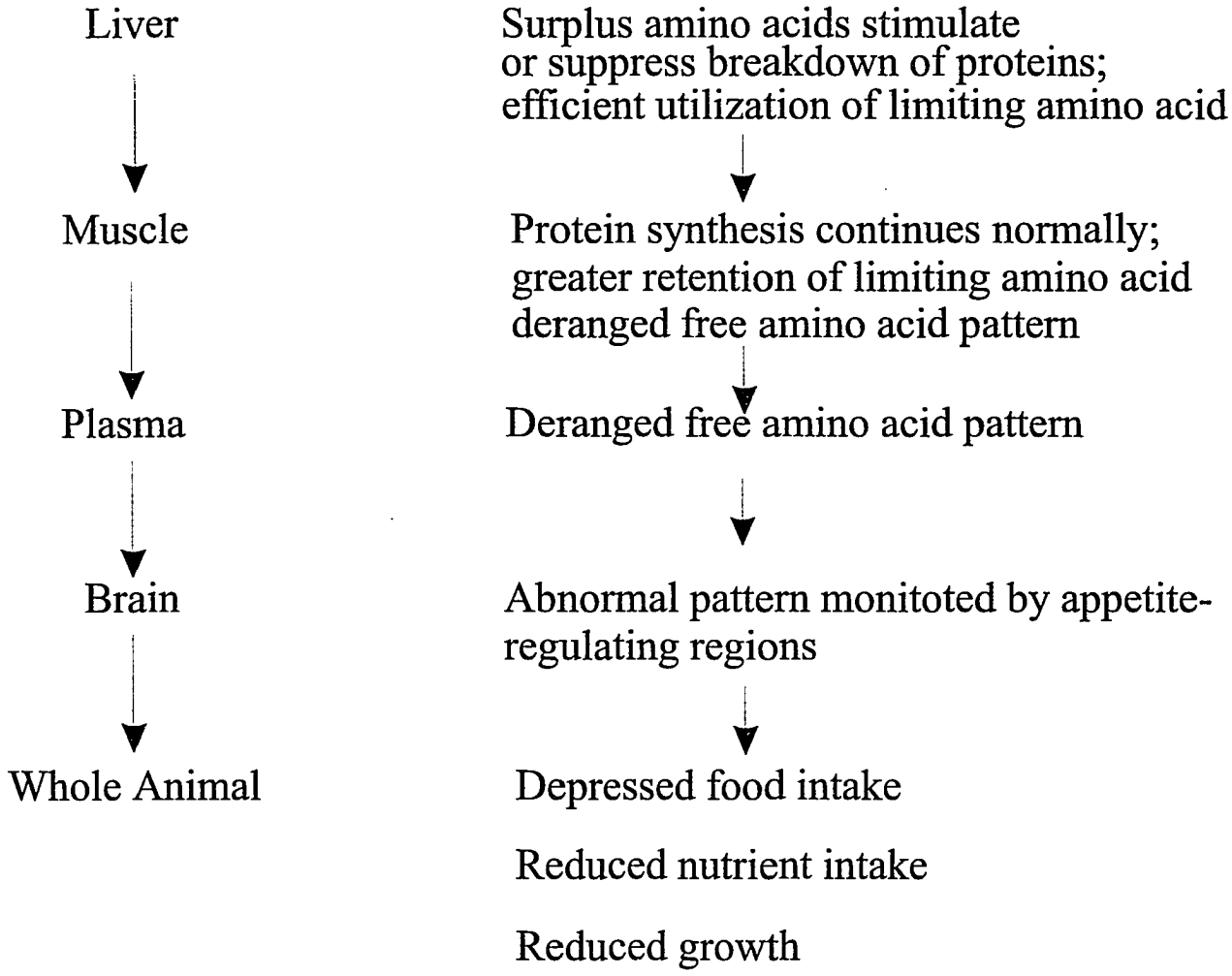
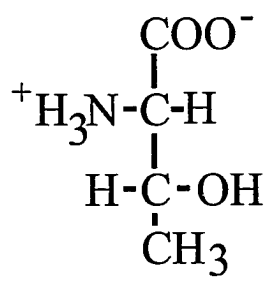
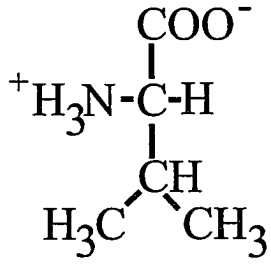


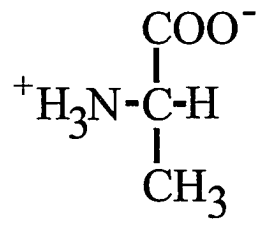
Figure 4.1, Effects of amino acid imbalance in the rat;
based on the hypothesis of Harper and Rogers (1965)



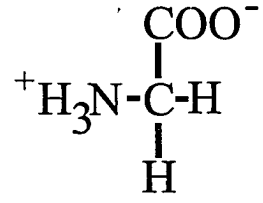
Threonine



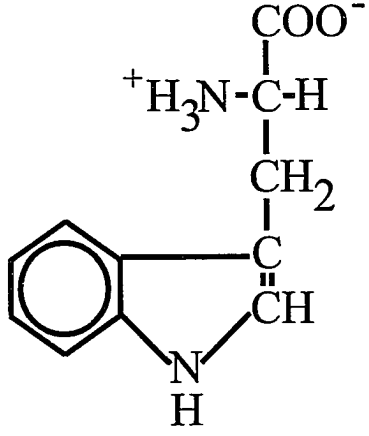
Valine



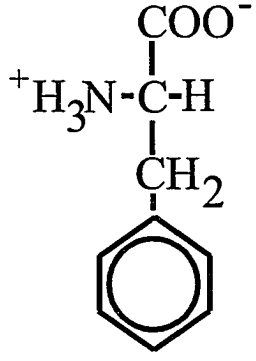
Alanine



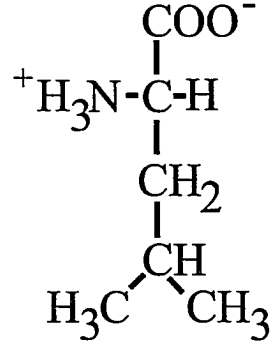
Glycine



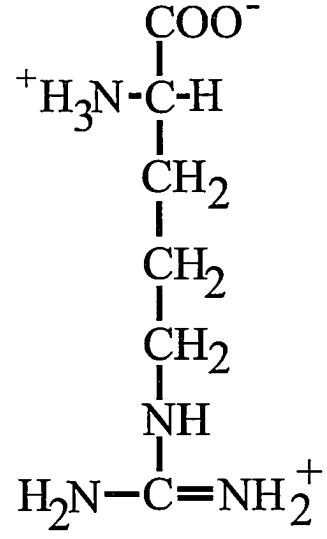
Tryptophan



Phenylalanine



Leucine



Arginine

Figure 4.2, Structures of the amino acids to be separated

Experimental

Amino Acid Standards

Amino acids were purchased from Sigma.(Sigma-DLAA). The separation procedure was obtained from a CAMAG application note (4.7) and involved separation of the following amino acids: alanine, tryptophan, glycine, arginine, threonine, phenylalanine, valine, and leucine. A solution containing the 8 amino acids was made by dissolving 0.05 g of each of the amino acids in 50 mls of distilled water. This solution was then dansylated. The dansylation procedure was to add 2.5 mls of the amino acid solution and 2.5 mls dansylchloride solution. This solution was allowed to dansylate for 16 hours in the dark. After dansylation, the solution was diluted to 25 mls with 7:3 acetone/water (v/v) using volumetric glassware. This was the stock solution used to make subsequent dilutions. The stock solution corresponded to 50 ng spots and dilutions of the stock solution were made ranging down to concentrations corresponding to 10 ng spots. Unknown samples of potatoes were prepared in similar fashion. Weighed portions of a potato were liquefied using a blender and 3.0 mls of this liquid was separated and to it added 10 mls of ethanol. The precipitated protein was filtered and the light brown liquid remaining was evaporated to dryness using a heating plate. This residue was dissolved in 3.0 mls of water and dansylation was performed on this solution as described previously.

Sample Application and Development

The amino acid solutions were applied to the plate using the modified spotter described in Chapter 2 with a flow rate of 2.5 μL/minute for 4 minutes at a regulator pressure of 28 psi. Spots were approximately 1.5 mm in diameter. The plates used for the separation were 5x10 cm, 200 μm layer thickness glass-backed Merck plates (Merck, Darmstadt, Germany). Plates were developed 9 cm beyond the origin with EDTA /n-

butanol/diethylether 1:2:7. The mobile phase was made up in the following manner: 5.0 grams EDTA (di-sodium salt) was weighed and transferred to a 100 ml flask. To the flask, 50 mls water was added and the pH adjusted to 9 using 1 N NaOH. To this, 10 mls n-butanol was added and the solution was vortexed. Next, 35 mls diethylether was added and the solution was vortexed again. The upper phase of the solution (organic phase) was extracted and used as the developing solvent.

Detector Configuration

System configuration was set up in the following manner. The scientifically operated PM512 (Photometrics, Tucson, Az.) was mounted on a stand in a vertical position, facing down. The system consisted of a CC200 camera controller and a CE 200 camera electronics unit. The camera was cooled to -100°C to prevent dark current accumulation. Exposure time was controlled with an electronic shutter mounted on the faceplate of the camera. On the shutter of the camera was mounted a f4.5, 105 mm focal length UV Nikor camera lens (Nikon) which functioned as the imaging optics. Spectral selection was accomplished by using a CVI Laser filter (CVI Laser Corporation, LWPS-400, Albuquerque, NM.). The illumination source used was the transilluminator system described in Chapter 3. Illumination of the plates was achieved by placing the silica surface of the chromatographic plates in direct contact with the UV pass filter (glass side up). Images were integrated for 30 seconds and were bias corrected. Images were flat fielded using a fluorescing filter (Y-48 Hoya Optics, Fremont, Ca.) as described in Appendix A.

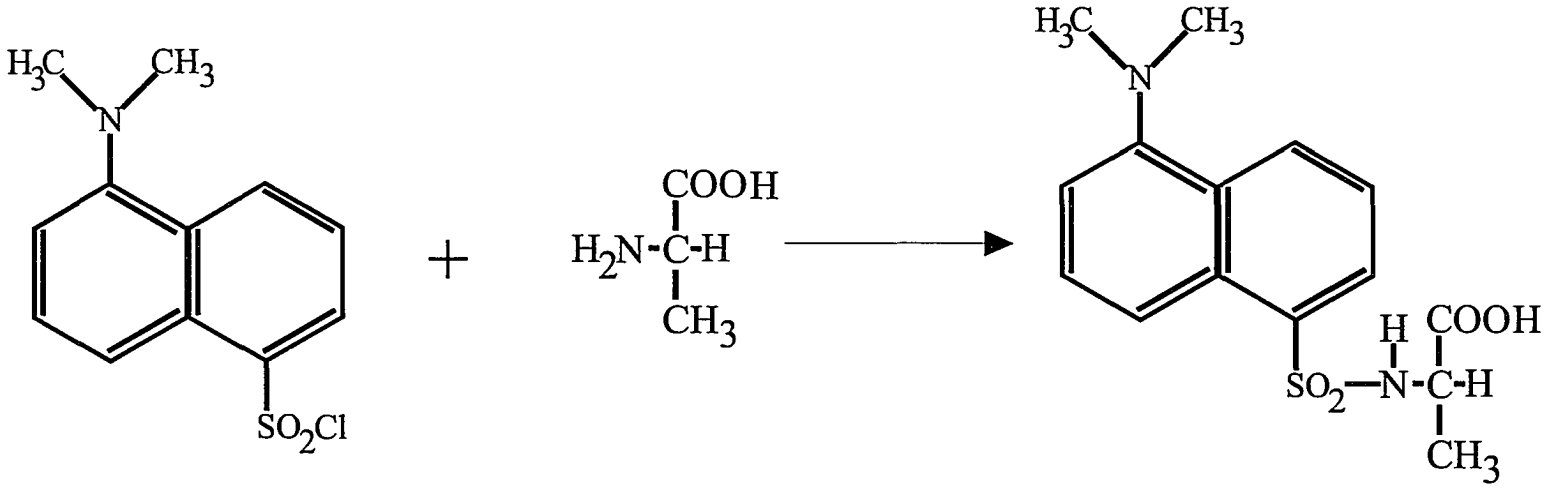


Figure 4.3, Dansylation of amino acid alanine

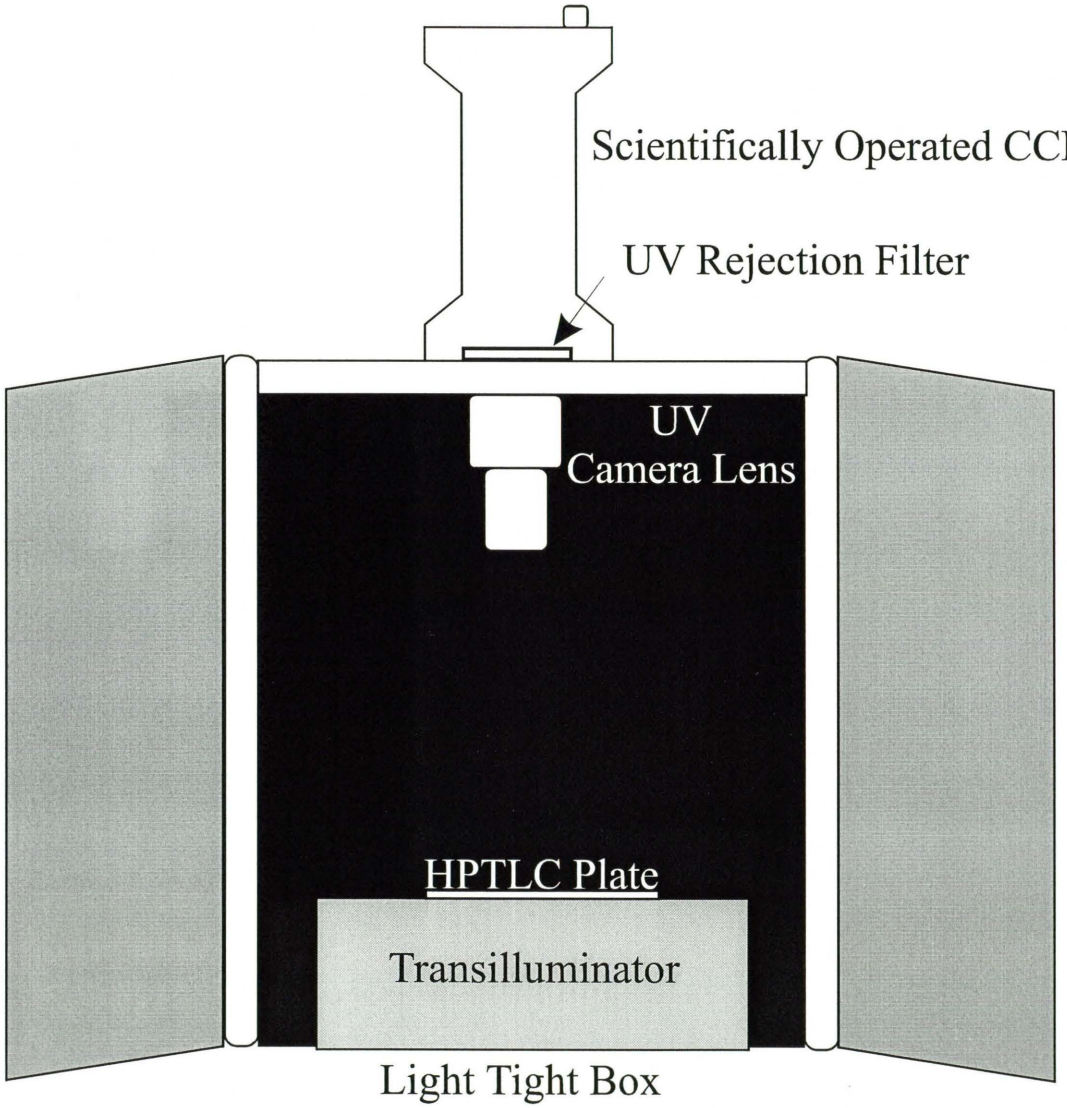


Figure 4.4, Amino acid detection arrangement

Results

Results for the detection and quantitation of the analytes was excellent. For the most part, the analytes were fairly well resolved. Not all of the peaks, however, were baseline resolved from nearby peaks. In these cases, peak height rather than peak area is used to generate calibration curves. It was also noted that the dansylated threonine and the excess unreacted dansylchloride peaks were difficult to resolve. This does not present a problem, however, since the unreacted dansylchloride peak and the dansylated threonine peak fluoresced at different wavelengths. The unreacted dansylchloride was more of a greenish-blue color compared to the dansylated amino acids, which were a yellowish-green. In an actual commercial application, the dansylated threonine could easily be quantitated by better spectral selection of detection wavelength, or by using peak height rather than integrated peak intensity.

The potato unknowns analyzed by the method differed significantly from each other in the number and quantities of amino acids, as shown in Figure 4.8.. In one of he unknown potato samples, large amounts of arginine were present but no other amino acids within the detection limits were found. There were also several unidentified peaks present not corresponding to anything in the known. The other unknown sample shows a considerably higher amino acid content of a variety of different amino acids. The quantitative amounts of the amino acids vary considerably from sample to sample as expected, validating the need to frequently analyze samples before consumption by livestock. In addition to the analysis of Russet brand potatoes, analyses were performed on similar products, including yams, red yams, sweet potatoes, and red potatoes. Results from the analyses showed a differing amino acid content of each of these products, although due to the limited number of samples analyzed, it is impossible to determine the most common amino acid pattern. It is likely that the content of amino acids found in the

different samples varies as widely as the content of the Russet potatoes. It was also noted that in nearly all unknown analyses, there was a large peak at the origin where the samples were applied. This non-migrating compound or compounds could pose problems for column analysis by irreversibly binding to the column and affecting all subsequent separations. This is a particular problem for column techniques when working with unknowns, since one never truly knows what is in the sample to a high degree of certainty. However, this is not a problem in an HPTLC medium, since all non-migrating species are easily seen at the origin, and will not affect future separation media.

Calibration curves for the amino acids were excellent, with R^2 values ranging from 0.975 to 0.999.

Detection limits for the amino acids are as shown in Table 4.1.

Amino Acid	Detection Limit
alanine	6 ng
arginine	1 ng
phenylalanine	1 ng
tryptophan	3 ng
valine	5 ng
leucine	5 ng
glycine	1 ng
threonine	5 ng

Table 4.1, Dansylated amino acid detection limits

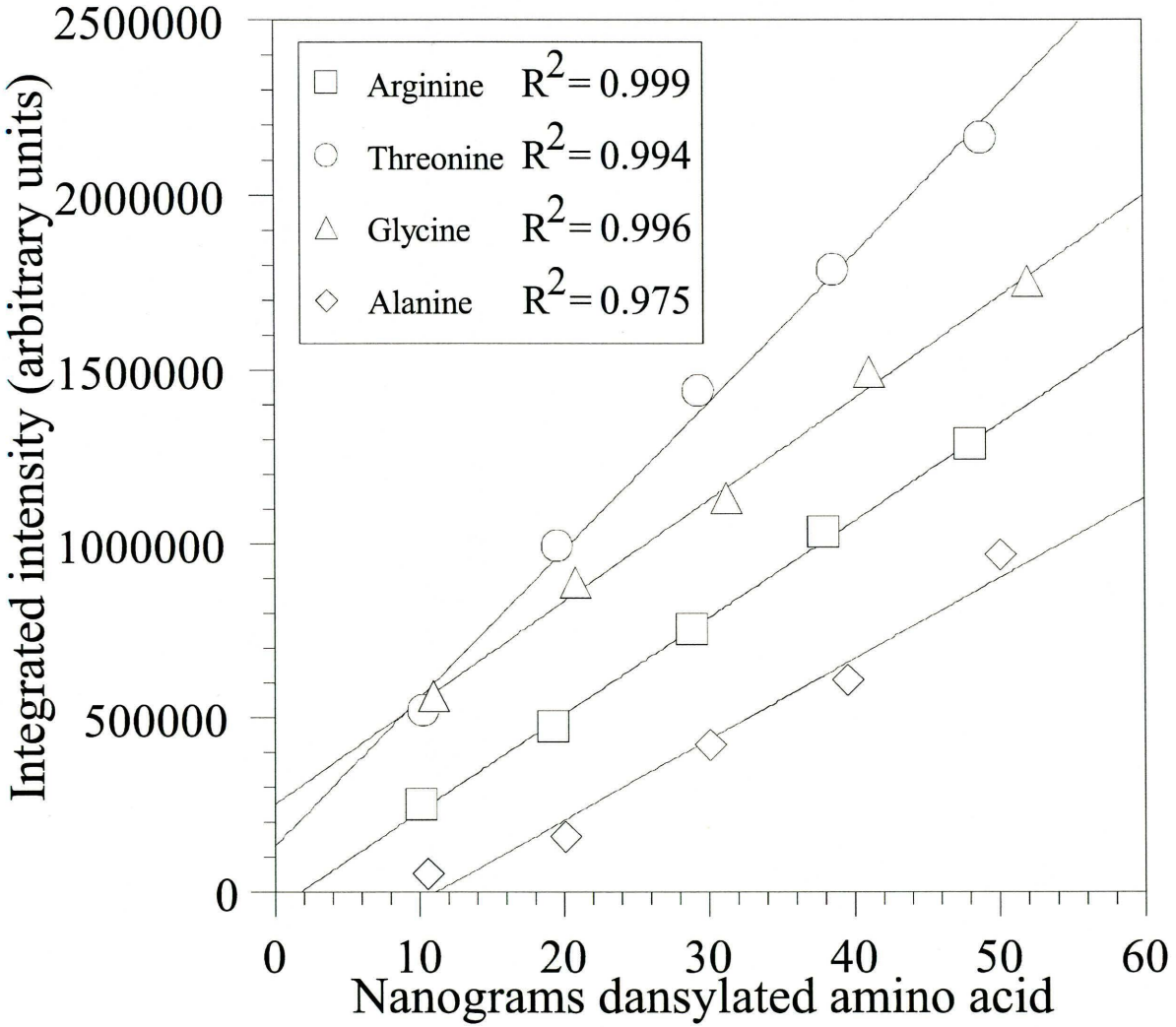


Figure 4.5, Calibration curve for dansylated amino acids arginine, threonine, glycine, alanine

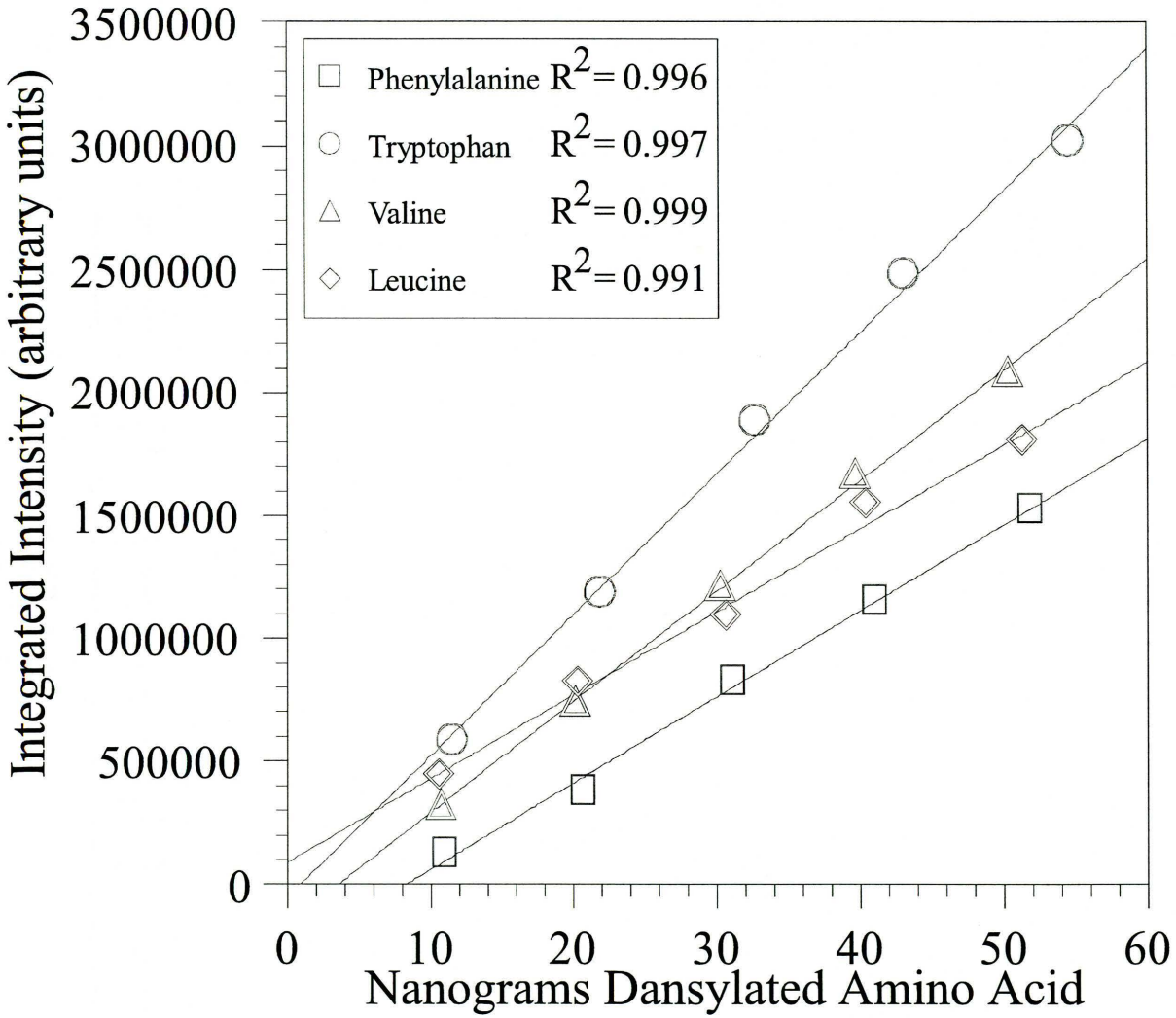


Figure 4.6, Calibration curve for dansylated amino acids phenylalanine, tryptophan, valine, and leucine

It was noted during the course of this investigation that the lifetime of the dansylated amino acids applied to the plate was very short. Typically, the spots would completely disappear within 30 minutes after the developing solvent was dried off. It can be seen in the CAMAG application note that this is compensated for by alternately applying standards and unknowns. This is to make sure the analytes haven't decayed to the extent that the calibration curve is invalid for the unknowns. At the rate the analytes disappear, the rate of scanning the plate must be very fast, or information will be lost. This is a severe drawback to the use of a scanning densitometer for this system. Analyte decomposition also limits the size of the plate that can be used for the separation. Often times, a 20x10 cm plate will be used to perform many analyses on the same plate. This would not be possible if using a scanning densitometer, since the most of the analytes would decay before they could be detected. The CCD imager allows the entire plate to be looked at simultaneously, eliminating the adverse effect of analyte decomposition and detecting all of the analytes immediately after the development solvent is removed, maximizing sensitivity and linearity of the analysis.

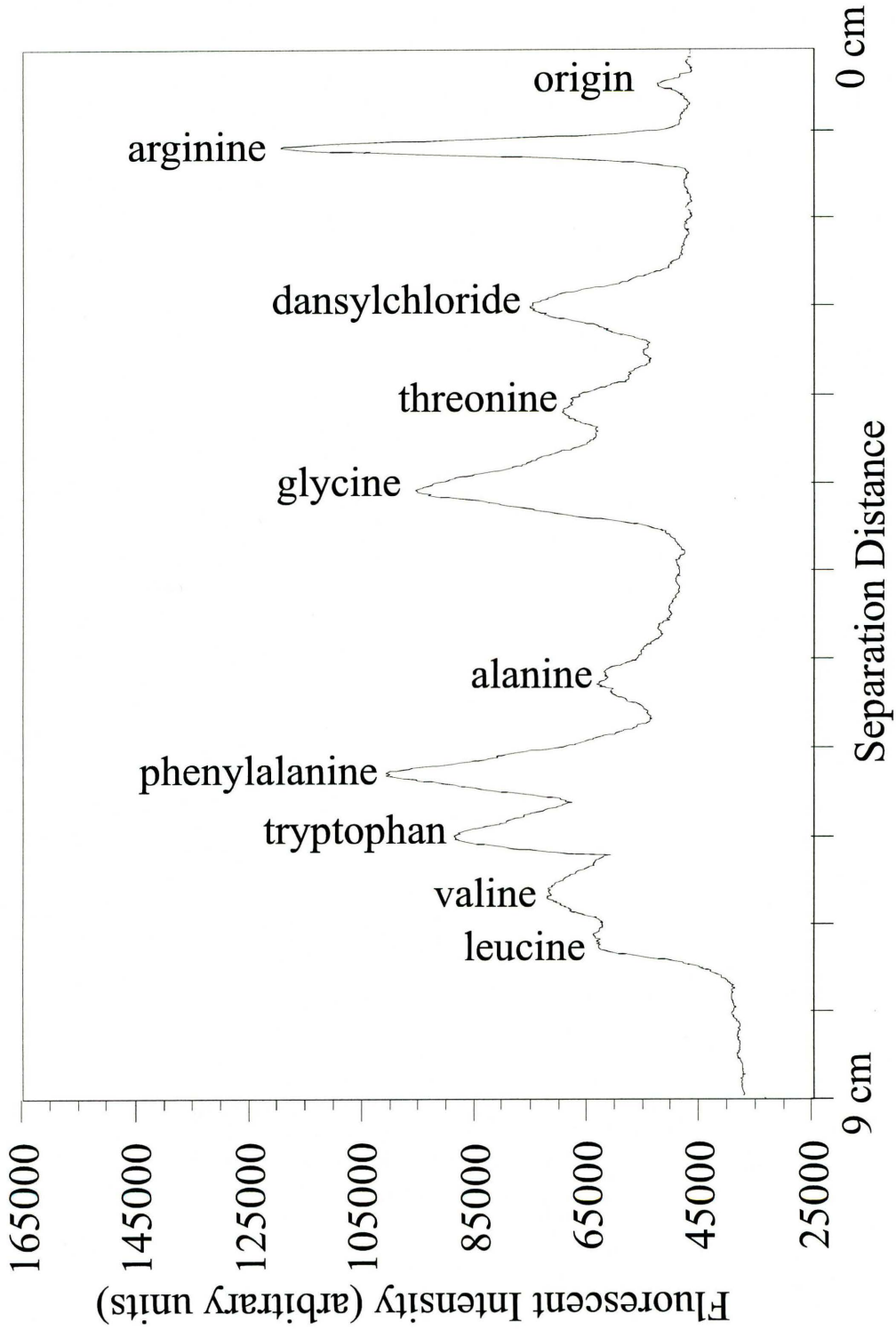


Figure 4.7, Separation profile of dansylated amino acids

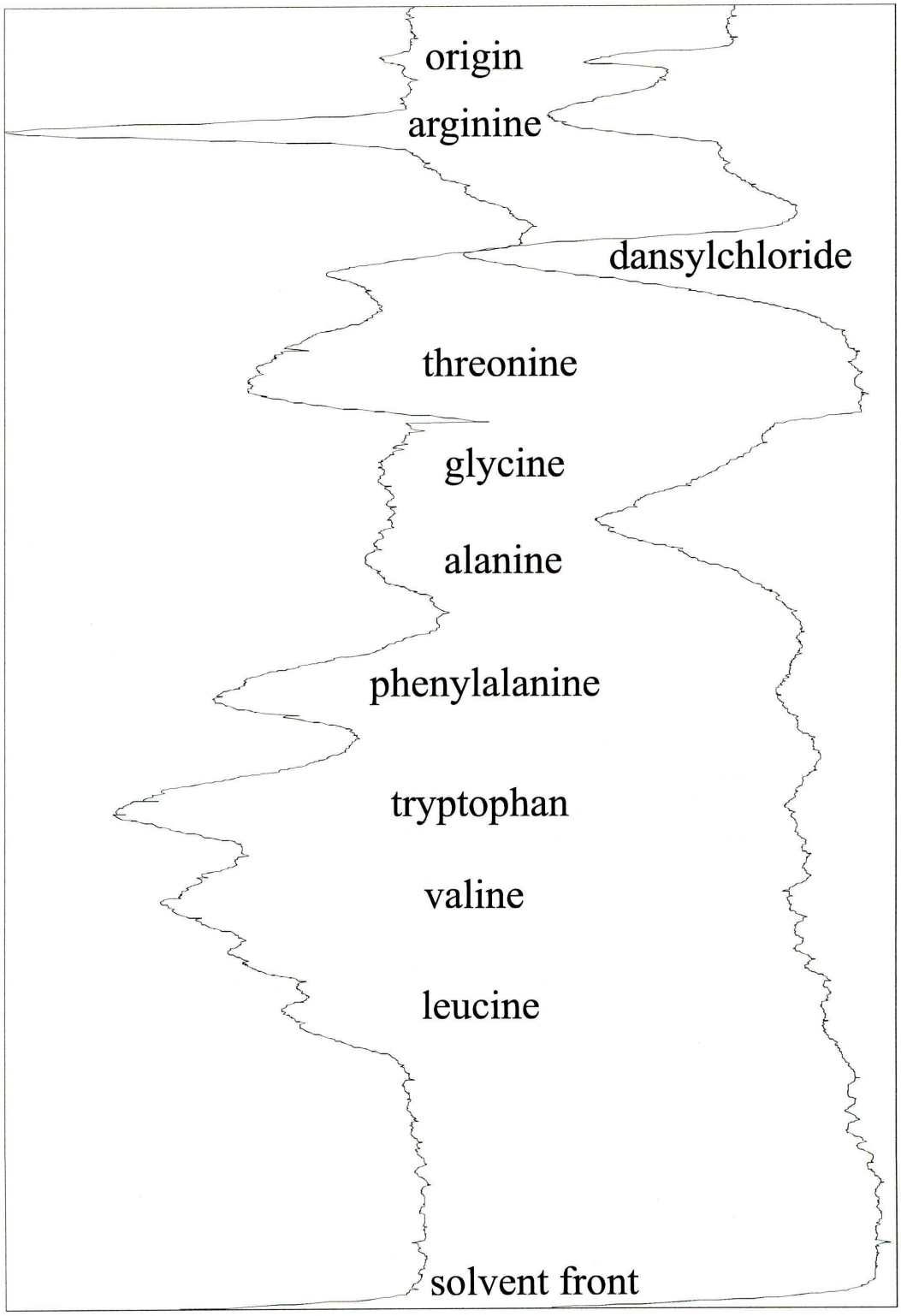


Figure 4.8, Amino acid content comparison of different potato unknowns

Overall, the advantages of array detection, disposable separation media, simultaneous analyte detection, low cost, simple unknown preparation, good quantitative analysis, and high sample throughput makes the analysis of dansylated amino acids by HPTLC an attractive technique.

Conclusions

The use of HPTLC combined with the imaging ability of the CCD is a viable technique to quantitate dansylated amino acids. The advantages of high throughput, simple sample preparation, good separation ability, and disposable sample media of HPTLC combined with the rapid analysis time, array detection ability, and excellent quantitative ability of the CCD makes the system an excellent choice for rapid and accurate quantitative analysis of amino acids.

CHAPTER 5

PRELIMINARY INVESTIGATIONS INTO THE USE OF A NEAR-INFRARED FOCAL PLANE ARRAY AS A DETECTION TECHNIQUE IN HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

Background

Near-Infrared (NIR) spectroscopy is a popular and useful technique that is commonly used in many industries. It is especially popular in the Foods industry, where it is used for such diverse applications as purity analysis, food quality, disease screening, quantitative and qualitative analyses of ingredients, protein content, and moisture analysis.(5.1) The reasons for its popularity are that cheap and robust materials, such as glass and quartz, are transparent to NIR radiation and that detectors in this region have rapid response times, so fast analyses are possible. The major absorbances in the NIR region are combination bands and overtones of the mid-IR range. The observed sample bands are derived from C-H, O-H, S-H, and N-H combinations/overtones . The most useful of these absorbances fall in the 2.0-2.5 micron region, where silica has essentially no absorbance.(5.2) Near-Infrared spectroscopy can also provide useful structural information for analytes of interest directly on the HPTLC plate.(5.3) In-situ NIR spectroscopy has been shown to be an effective, non-destructive detection scheme, providing quantitative and structural discrimination of similar compounds directly on the HPTLC surface without modification of the silica surface or removal of the analyte. Frey, Kovar, and Hoffman (5.4) studied caffeine on a HPTLC plate from 2.5 to 10 microns and discussed problems and sources of error by using circular apertures as opposed to slits in scanning the plate. Yamamoto, et al (5.5) studied the spectra of

various phospholipids and performed quantitations directly on a HPTLC plate using a Fourier-Transform Infrared Spectrometer (FTIR) as a scanning densitometer. Ciurczak, Cline-Love, and Mustillo (5.6) have studied amino acids and other compounds on a HPTLC plate using a Near-IR spectrometer and have performed spectral matches against known compounds to determine the discriminatory power of the unit. They found that amounts as low as 4 micrograms could be detected and quantitated directly on the plate without visualization or derivitization. They also noted that around 1900 nm, the maximum absorbance was due to the Si-OH band from the stationary phase, and that analytes spotted on the plate caused the Si-OH absorbance to diminish in proportion to the amount of analyte. At 2280 nm, they noted it was the C-H combination bands of the analyte that predominated, and that as the concentration of the analyte increased, the peak absorbance increased. The implication was that one may focus on the 1900 nm region and have a universal detector based on Si-OH lowering, or focus on the organic portion of the spectrum for more specificity. All of the NIR studies were done by scanning the plate in a manner identical to visible scanning densitometry using a single channel detector in either reflectance or transmittance mode. While the results of these experiments are useful, the major advantage of HPTLC, namely throughput, is still not taken to its full advantage. The detection scheme is still limited to a single channel, focusing on one spot at a time. This detection mode, while providing structural information of the analytes on the plate, does not take advantage of the parallel nature of the HPTLC medium. Analyte detection is still the limiting step in the throughput of the system.

The goal of this study is to prove the feasibility of the use of a multi-channel NIR focal plane array as a rapid array detection technique and evaluate the performance of the array as a detection technique for a HPTLC medium.

Near-Infrared Spectroscopy

Infrared Focal-Plane Arrays

One of the disadvantages of silica based CCD's is the inherent limitation of wavelength response in the infrared region of the spectrum. The bandgap of silica at approximately 1.1 microns makes detection of longer wavelengths impossible. For detection of these infrared wavelengths, different detector materials must be employed. It is in this region that infrared focal plane arrays are useful. Infrared focal-plane arrays have made significant advances in recent years, and with the de-militarization of many industries, infrared focal plane arrays have become more available and less expensive. There are many different configurations of infrared detectors, as shown in Table 5.1

Type	Spectral Range (μm)	Availability
Ge	0.8-1.6	Commercial
InGaAs	0.8-1.6	Commercial
PtSi	1-5	Commercial
IrSi	1-8	Prototype
InGaAsP	0.8-2.6	Prototype
InSb	1.0-5.5	Commercial
Doped-Si BIB	1-28	Prototype
Hybrid MCT/Si	2-12	Prototype

Table 5.1, Different Types of Multichannel Imaging Detectors

Chase, B. "Arrays for Detection Beyond One Micron", in **Charge Transfer Devices in Spectroscopy**, Sweedler, J.V., Ratzlaff, K.L., and Denton, M.B. eds., VCH Publishers, 1994 pg.113

These focal plane arrays have been used previously in a wide variety of applications, ranging from astronomy to thermal monitoring and are undergoing explosive growth as the unit costs decrease and performance increases. It is beyond the scope of this work to detail operations of all of these focal plane arrays, so attention will be focused on the array type used in this chapter, the indium-antimonide focal plane array.

Indium Antimonide Focal Plane Array

Two-dimensional Indium Antimonide (InSb) arrays have existed since the late 1970's and were typically used in military applications, such as missile tracking, thermal sensing, and night-vision applications. Recently, however, InSb arrays have become increasingly available for commercial applications. The active spectral response region for indium-antimonide covers the regions from 1.0 to 5.5 microns, with quantum efficiencies as high as 70%. The detector is not monolithic, meaning the active detection region is manufactured from a different material than the multiplexer. In the case of indium-antimonide, the photoactive region is composed of indium-antimonide, which is Indium bump-bonded to a silicon multiplexer, which performs readout functions. Currently, arrays are available in sizes up to 256x256 pixels, with each pixel being 43 microns square. The arrays are operated in photovoltaic mode. In operation, the InSb detector elements are zero-biased and operated in short circuit current mode. The multiplexer input structure provides a low-impedance current sink into a capacitor. This capacitor is reset to initiate each image frame acquisition. When NIR photons strike the focal plane array, photogenerated current is collected through the input structure and integrated for a operator defined amount of time. The integrated signal is limited to the full well capacity of approximately 50 million carriers. Upon signal integration, the silicon CMOS multiplexer addresses each element on a row-by-column basis, presenting

the signal voltage in a single output line for A/D conversion. After signal readout, the capacitor dumps its charge well capacity and frame integration is repeated. Typical integration times range from as little as 1/1000 sec to 1 frame/sec. In operation, the focal plane array is cooled to 77K to minimize thermal noise. Thermal noise is typically the limiting noise factor and limits effective integration time. While InSb detectors are inherently capable of >16-bits of total dynamic range, typical dynamic ranges are only 12-bits, owing to the speed at which the arrays are often read-out.

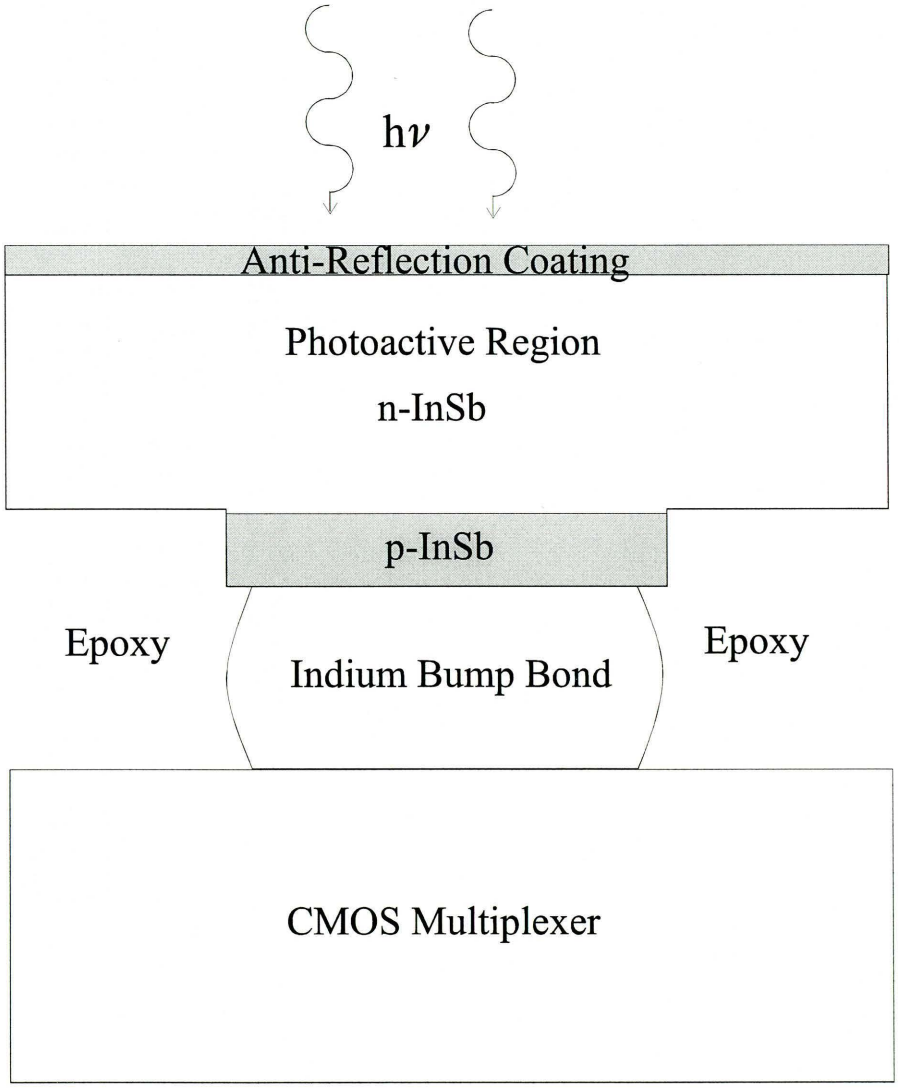


Figure 5.1, Schematic diagram of the InSb detector structure

System Configuration

NIR focal plane array

In cooperation with Amber Engineering, a 128x128 Indium Antimonide (InSb) focal plane array was used to perform feasibility studies on the in-situ array detection of analytes on the HPTLC plate.

The infrared camera used was an Amber AE-4128 indium-antimonide camera system. The camera has a response range from 1 to 5.5 microns and quantum efficiencies of 40% over most of the response range. The 16384 individual detectors are each 43 microns across on an even 50 micron pitch. The camera uses a 12-bit 2 MHz A/D converter and integration times can be adjusted between 1 and 217 frames/second. Integration times for this study were 1/40 sec.

For the chemical analysis, the optical arrangement was set up in both transmittance and reflectance modes, to help determine the flexibility of the instrument and optimum configuration. Two different systems were studied to test the feasibility of using the array detector for infrared detection on a HPTLC plate.

Benzocaine System

The first system attempted to reproduce the work of Mustillo and Ciurczak.(5.7) Sample Application: Varying amounts of benzocaine were spotted on Merck HPTLC plates with 200 μm layer thickness and glass backing. Spots were applied using the modified nebulizer head described in Chapter2. Application times were 6 minutes at a flow rate of 2 μL/min with a regulator pressure of 26 psi. Glass-backed Merck brand HPTLC plates with 200 μm thick silica were used in this study.

System Configuration: The system was setup in reflectance mode as shown in Figure 5.2. The source is a tungsten filament lamp operating in constant voltage mode. The reflecting mirror redirected the light to fall on the silica side of the HPTLC plate, that had the benzocaine spotted on it. The InSb camera was run at 77 K with a 1.95 micron centered, 0.065 micron FWHM bandpass filter employed to reject unwanted wavelengths. Flat field images were taken and saved prior to spots being placed on the plate. After the spots were applied to the plate, another picture was taken, and the flat field image was corrected for to obtain the final image. Images were obtained using the AmberView software package packaged with the system. Images were processed using a Labview based program to integrate the intensity of the spots.

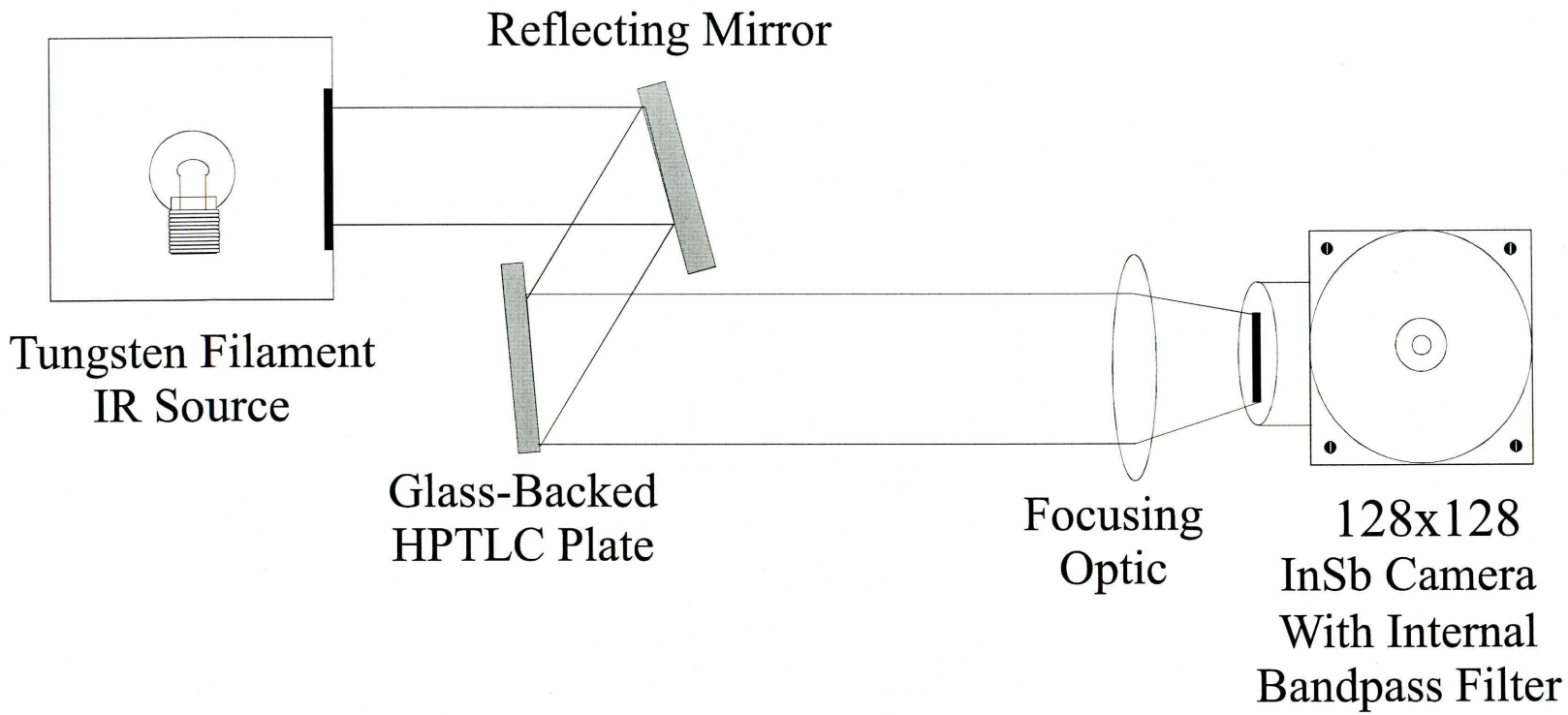


Figure 5.2, Benzocaine system configuration

Phosphatidylcholine System

The other system studied attempted to reproduce the work of Yamamato et al.(13), who studied phosphatidylcholine on silica HPTLC plates utilizing a Fourier-Transform infrared spectrometer as a scanning densitometer to obtain quantitative and qualitative information of the analytes studied.

Sample Application: Various amounts of Phosphatidylcholine, which has no usable absorbances in the visible or UV (5.8) were spotted onto a 200 μm layer thickness Merck brand silica HPTLC plate with glass backing. Spots were applied using the modified sample applicator described in Chapter 2. Application time was 6 minutes with an application rate of 2 μL/min.

System Configuration: The camera was fitted with a 2.483 micron centered, 2.092% bandpass filter to reject unwanted wavelengths. The system was operated in transmittance mode, at 77K with the silica side of the HPTLC plate facing the camera. Flat fields were again taken and corrected for in the final image to obtain a picture for analysis. The system was setup as shown in Figure 5.4.

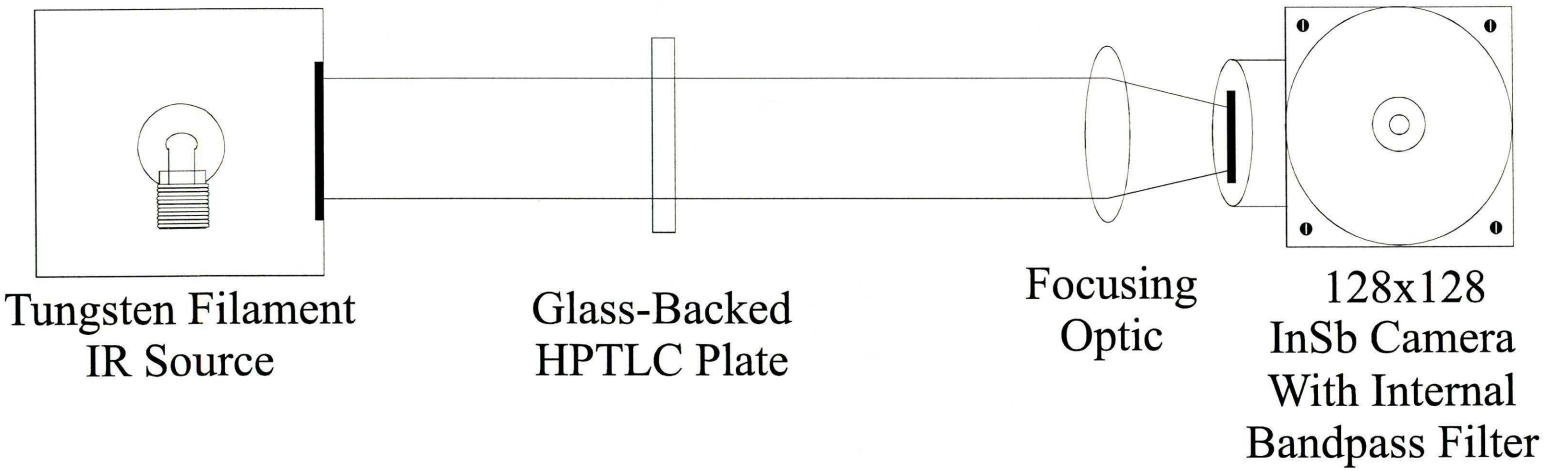


Figure 5.3, Phosphatidylcholine system configuration

Results

Benzocaine System

Calibration curves were generated for the benzocaine system, as shown in figure 5.5. The results shows excellent sensitivity and linearity, with an R^2 value of 0.993. The results obtained also show excellent agreement with results obtained by use of a single-channel NIR detector, and the images were obtained in a fraction of the time that scanning densitometry requires. Twenty microgram spots of Benzocaine were easily observed and the actual detection limit was determined to be well below 1 μg, though no rigorous detection limit studies were performed due to time constraints. The phenomena noted by Mustillo and Ciurczak, that around 1900 nm, Si-OH absorbance dominated, and analyte absorbance decreased as concentration increased, was also noted in our studies. This caused the spots to be seen as light spots on a dark background. The phenomenon appeared to be linear over the extent of these studies. This phenomenon could form the basis of a Universal detector. If detection were performed at 1900 nm, any analyte on the plate could be detected and quantitated by noting the amount of Si-OH lowering. This is an important function in a HPTLC medium. It would allow the operator to detect the presence of all analytes on the plate. With this information, further analysis of unexpected spots or spots not corresponding to known analytes could be performed at different wavelengths to obtain more specific information about the analyte.

Phosphatidylcholine System

Calibration curves were generated for the phosphatidylcholine system, as shown in Figure 5.6. The results show excellent linearity, with an R^2 value of 0.992, as well as good agreement with previous studies using FTIR scanning densitometry. Spots appeared as dark areas on a light background. Detection limits appeared to be about 500 ng, though no rigorous detection limit studies were performed due to time constraints.

The images were again obtained in a fraction of the time required by scanning densitometry.

An additional experiment was performed to test the discrimination ability of this configuration. A spot of benzocaine was laid down next to a spot of phosphatidylcholine using the 2.483 micron bandpass filter, configured as above. The benzocaine has almost no absorbance at 2.483 microns while the phosphatidylcholine has a very strong absorbance in that region. The image obtained showed that only the phosphatidylcholine could be seen, appearing as a dark spot on a light background, while the benzocaine spot showed no discernible difference from the background. This illustrates the spectral discrimination power of the system and can be employed to obtain excellent qualitative information. This, combined with the universal detection mode at 1900 nm, could form the basis of a very powerful detection technique for HPTLC. These experiments show the feasibility of a detection system capable of obtaining qualitative and quantitative information on all analytes present on a HPTLC plate without removal of the analyte from the plate, and in a very short time when compared to conventional techniques.

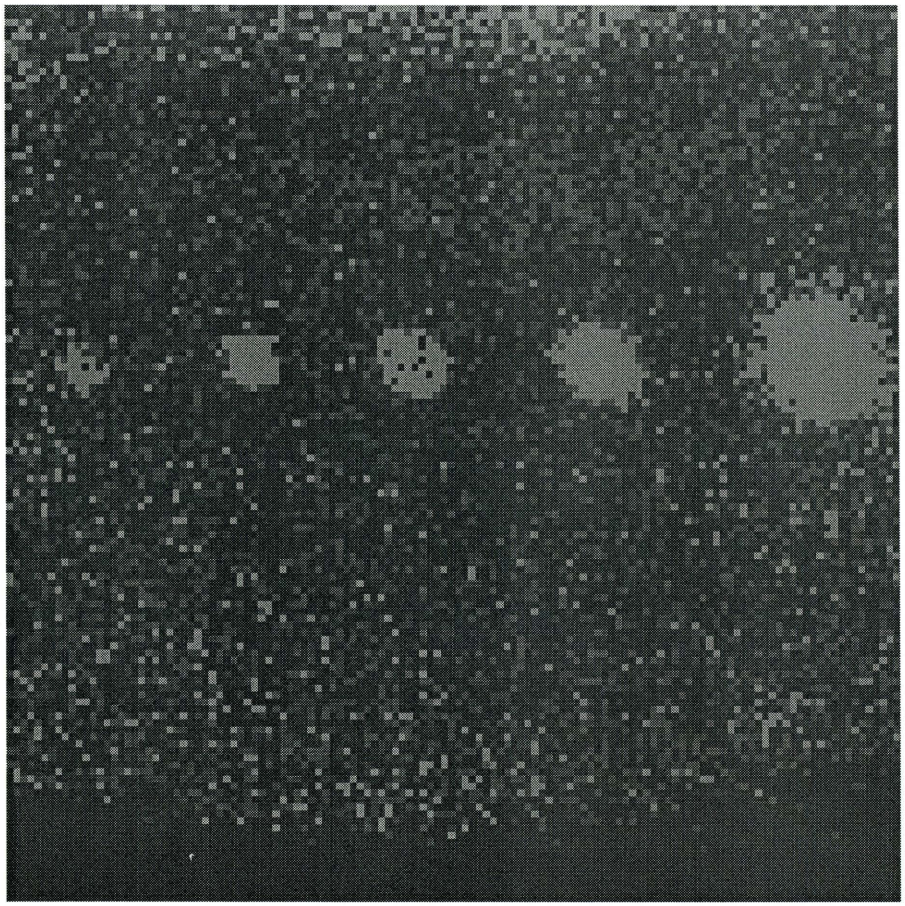


Figure 5.4, Image of varying concentrations of phosphatidylcholine obtained at 2.483 microns with the InSb focal plane array

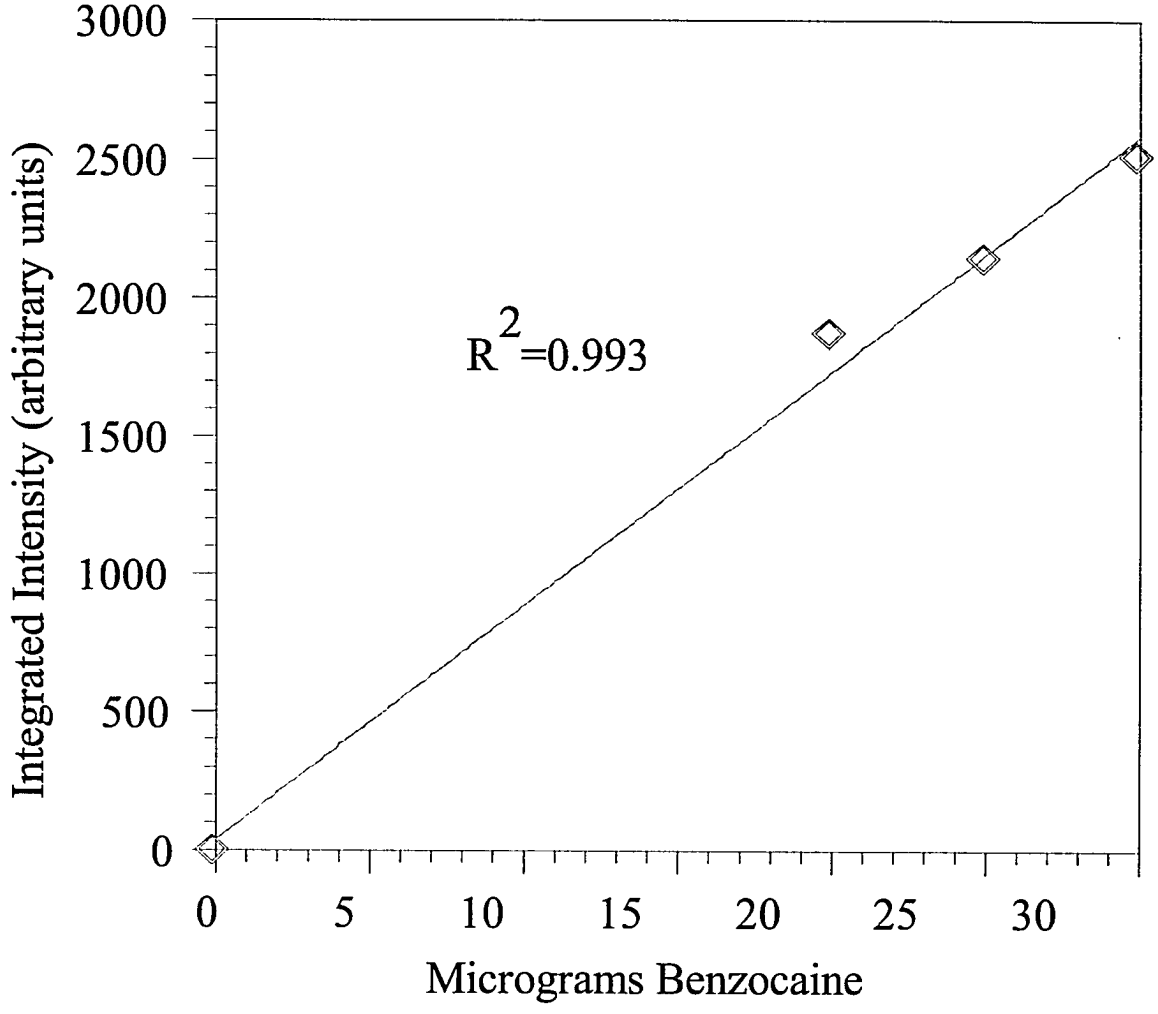


Figure 5.5, Calibration plot of benzocaine obtained at 1.9 microns

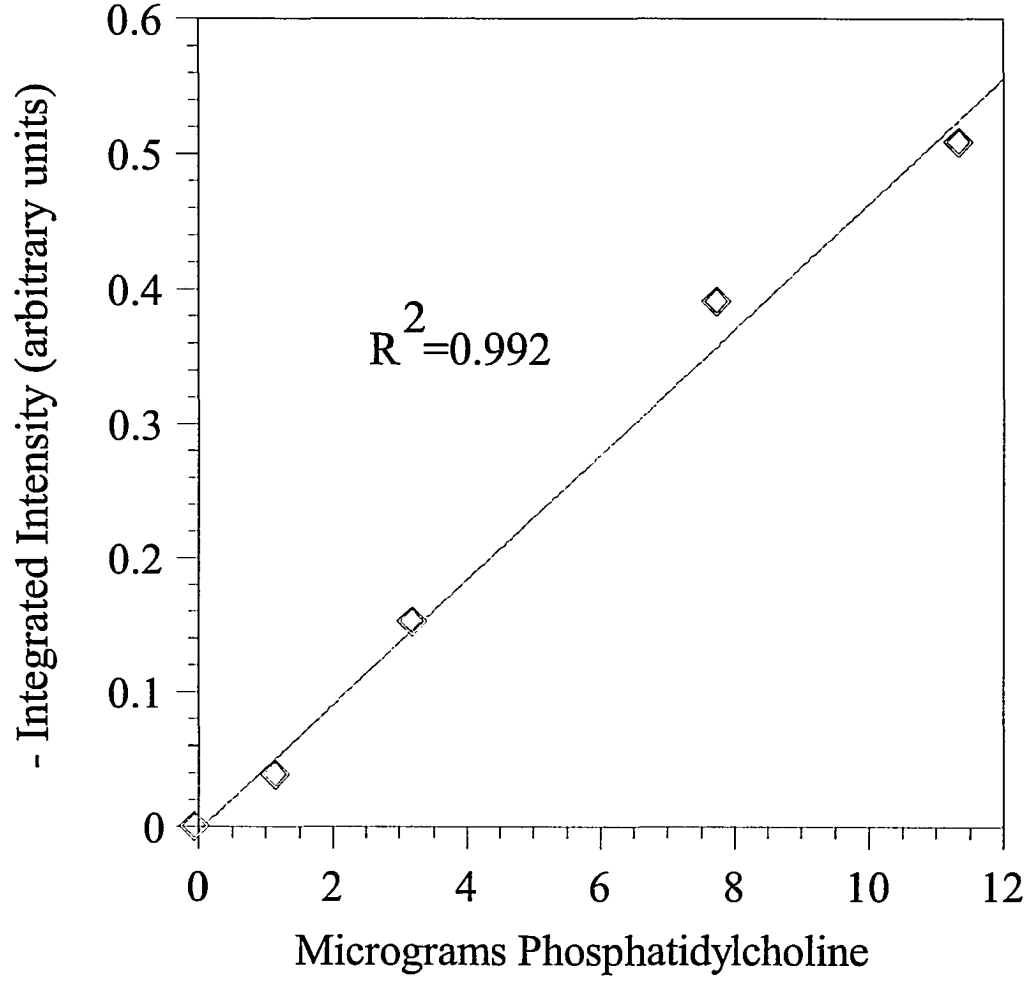


Figure 5.6, Calibration curve of phosphatidylcholine system taken at 2.483 microns

Conclusions

As the previous demonstrations show, the combination of HPTLC with Near-Infrared imaging spectroscopy are well suited to each other. The combination of the planar format, separation power, low cost per sample, static detection, and throughput of HPTLC combined with the spectral coverage, spectral discrimination, system flexibility, good quantum efficiencies, and imaging capabilities of the InSb focal plane array, creates a powerful separation and detection system. A system that can separate, quantify, and positively identify a very large numbers of samples in a very short time, and, with the anticipated decrease in costs of infrared focal-plane arrays, at a reasonable cost. Additionally, the flexibility of the system to run in either reflection or transmission mode, and cover wavelengths from 1.0 to 5.5 microns makes the unit an extremely useful and powerful HPTLC detection system. We anticipate that a commercial version of the system would be further optimized to give lower backgrounds, better sensitivity, and lower detection limits. We envision a system that ultimately eliminates the filters for spectral selection and incorporates an Acousto-Optic Tunable Filter (AOTF) as both a source and spectral selection unit. The AOTF is capable of sweeping through wavelengths very rapidly, on the order of microseconds per change. An AOTF also has a very narrow bandpass, between 2 and 10 nm, allowing it to very accurately select a particular absorption band in the near-infrared. The combination of the high speed abilities of the InSb focal plane array, combined with the rapid wavelength selection of the AOTF, will allow an extremely powerful HPTLC detection unit. This unit would be capable of acquiring the near-infrared spectrum of every analyte on the HPTLC plate and quantitate in a matter of seconds. Also, as infrared focal-plane technology becomes more mainstream, initial costs will decrease and detector performance will increase, as has been the case with standard CCD's.

CHAPTER 6

USE OF A HOME VIDEO CAMERA AS AN INEXPENSIVE ARRAY DETECTION TECHNIQUE IN HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

Introduction

One of the primary advantages of high performance thin layer chromatography is the low cost of performing a separation. There is no expensive equipment required to do most separations, since the separations occur on inexpensive glass-backed silica plates. This is a primary reason for making the decision to do a particular separation on a HPTLC medium. This advantage, however, is often nullified by the cost of the detection and quantitation equipment. The detection system to analyze the separation can cost as much as an entire HPLC separation system, causing many researchers to choose the column-based separation system. There are typically three options a researcher has in choosing a quantitation system for HPTLC. The first choice involves the use of visual inspection with known samples, to get a semi-quantitative estimation of analyte concentration. This technique, though inexpensive, is simply inadequate for even semi-quantitative analysis of analyte concentration. Another option is to use an instrumental system, such as a scanning densitometer or CCD array detector. Although giving excellent quantitative information, these systems are expensive, costing as much as $20,000-$30,000 dollars. Additionally, the scanning densitometer is extremely slow and greatly reduces the throughput of HPTLC, one of its major advantages over the column techniques. The third option is to use an off-line detection method, such as Fourier-

Transform infrared spectroscopy (FTIR) or gas chromatography (GC) as a quantitation technique. These detection means, while giving good quantitative results, are also very expensive and often require the sample be scraped off the plate to be analyzed, again limiting the throughput of HPTLC. This leaves the researcher in the difficult position of either paying for an expensive detection system to get good quantitative results, or using an inexpensive detection system and obtaining only semi-quantitative results. Both of these options are undesirable at best, and another option is needed that combines low cost, quantitative results, and fast analysis time. The solution to this problem is to use an inexpensive array detector that many people have in their homes, the video camcorder. By using a video camera with its CCD array, combined with an inexpensive frame grabber to obtain an image of the plate, an extremely low cost, high speed, quantitative analysis system is created that is extremely simple to use. The system was designed to be operated by a wide variety of users, from a high school student to the analytical scientist with little or no change in equipment or cost. This chapter describes the system and results obtained by using a video camera as a detector in three modes; visible absorbance, fluorescence quenching, and fluorescence. It also describes sample separations designed to test the ability of the system to obtain quantitative results, and makes comparisons to the scanning densitometer and the scientifically-operated CCD.

System Configuration

The system was tested in a variety of means in order to determine its applicability to a variety of tasks. The different system configurations were 1) visible absorbance in reflectance mode using tungsten lamp illumination as well as room light illumination, 2) fluorescence quenching using a mercury vapor lamp in reflectance mode, and 3)

fluorescence using a transilluminator in transmission mode. The individual system setups are described in the following sections.

Visible Absorbance System Configuration:

The first system configuration to be tested was visible absorbance in reflectance mode. The system was configured as shown in Figure 6.1. The video camera (JVC Model GR-AX33, Compact VHS) was mounted on a post facing the silica side of an HPTLC plate. The plate was mounted in an adjustable square filter holder approximately 6 inches from the front of the video camera. When using tungsten lamp illumination, a normal tungsten desk lamp illuminated the plate from the front in reflection mode approximately 12 inches from the plate face. For room light illumination, no additional light source was used. For spectral selection, various color Tiffen filters (Tiffen, Manufacturing Corporation, Hauppauge, NY) were mounted on the front of the camera. The camera was setup in manual focus mode. Images were obtained using a Snappy Video Snapshot frame grabber (Play Inc.,), connected to a serial port of a Windows-based PC.

Fluorescence Quenching System Configuration

The system configuration for fluorescence quenching was similar to the visible absorbance system configuration. The only change in system configuration involved replacing the tungsten filament bulb with a mercury vapor lamp, emitting at 254 nm. The only other change was the use of pre-coated fluorescent plates (Merck, F254 Kieselguhr 60, Art. 5635) that emitted in the green portion of the spectrum when illuminated by 254 nm light. The Tiffen filter was replaced with a cut-off filter designed to eliminate all Ultraviolet light. This ensured that all photons being detected were fluorescent photons

from the HPTLC plate. All quenching experiments were performed under darkened room conditions. The system is illustrated in Figure 6.2

Fluorescence Detection System Configuration:

The setup for doing fluorescence detection is similar to the setups previously described. In this mode, the illumination source is a UV transilluminator as described in Chapter 3, placed directly behind the HPTLC plate, as shown in Figure 6.2. The filter is the same UV-cutoff filter used in the fluorescent quenching mode. An analyte was chosen that would fluoresce when illuminated by 380 nm light, namely Rhodamine-6-G. The HPTLC plates used (Merck Kieselguhr 60, Art.5644) were not pre-coated with fluorescent indicator. Plates were oriented with the silica side facing the camera. All fluorescence experiments were performed under darkened room conditions. The system is illustrated in Figure 6.3.

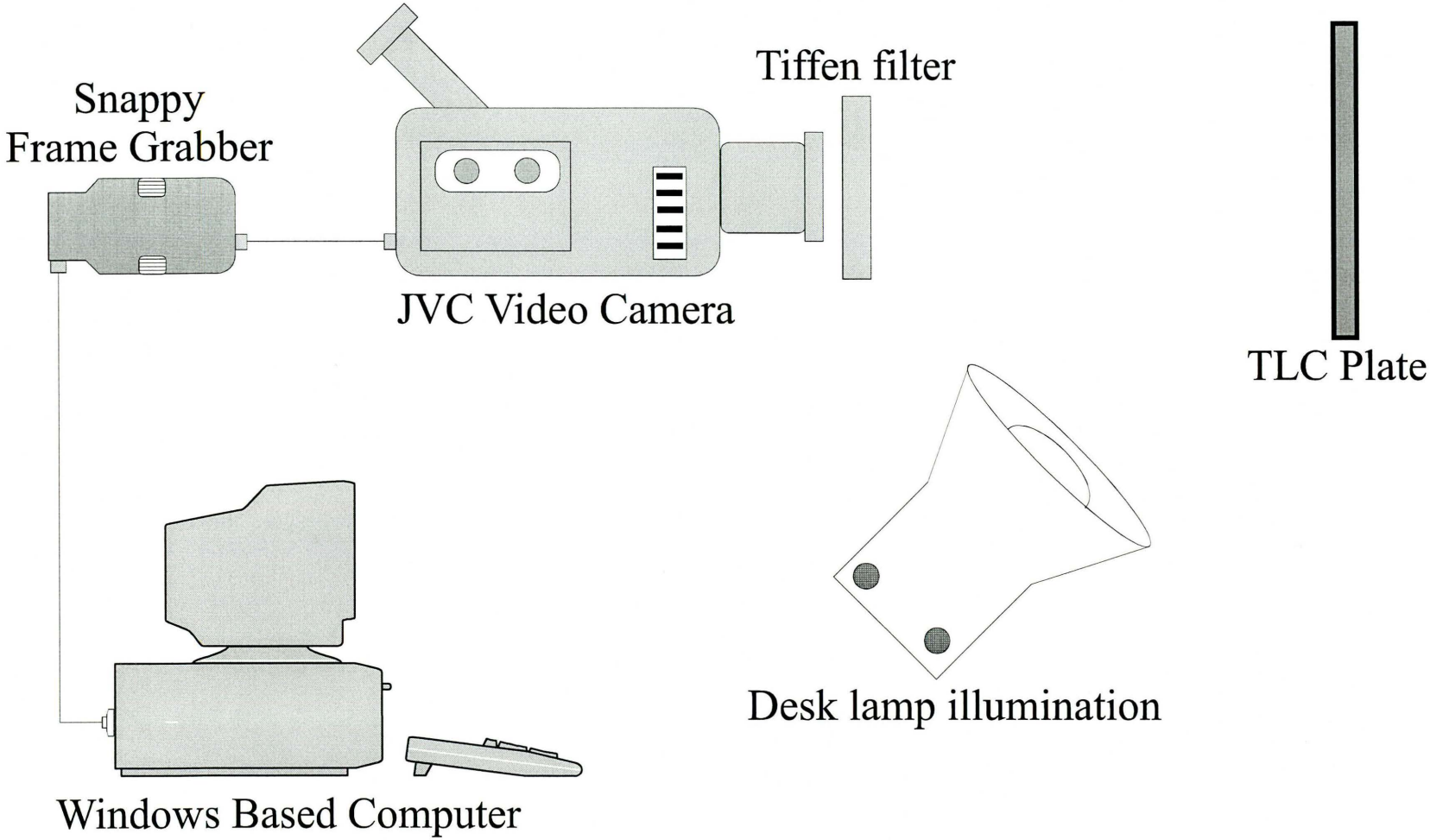


Figure 6.1, Visible absorbance system configuration

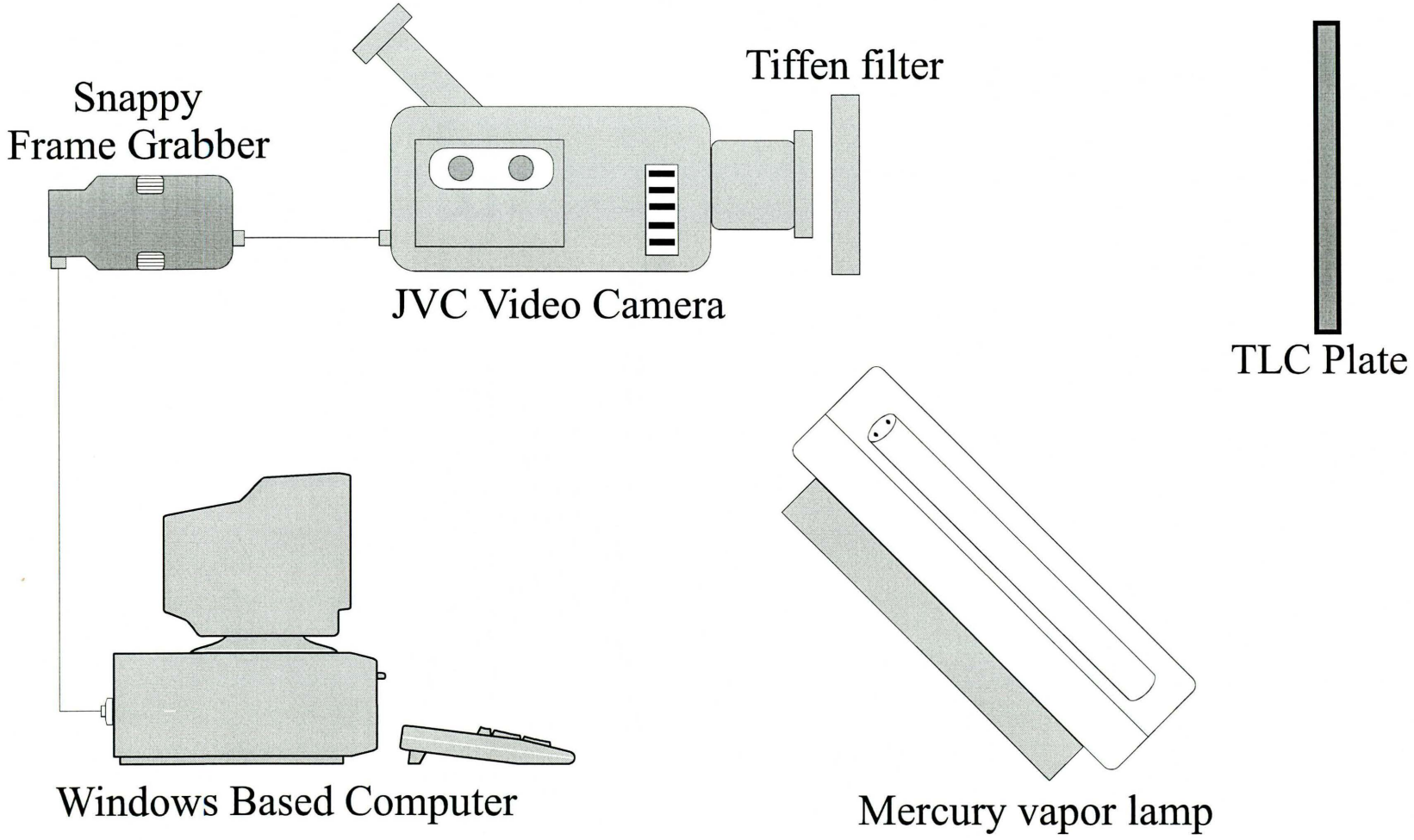


Figure 6.2, Fluorescence quenching system configuration

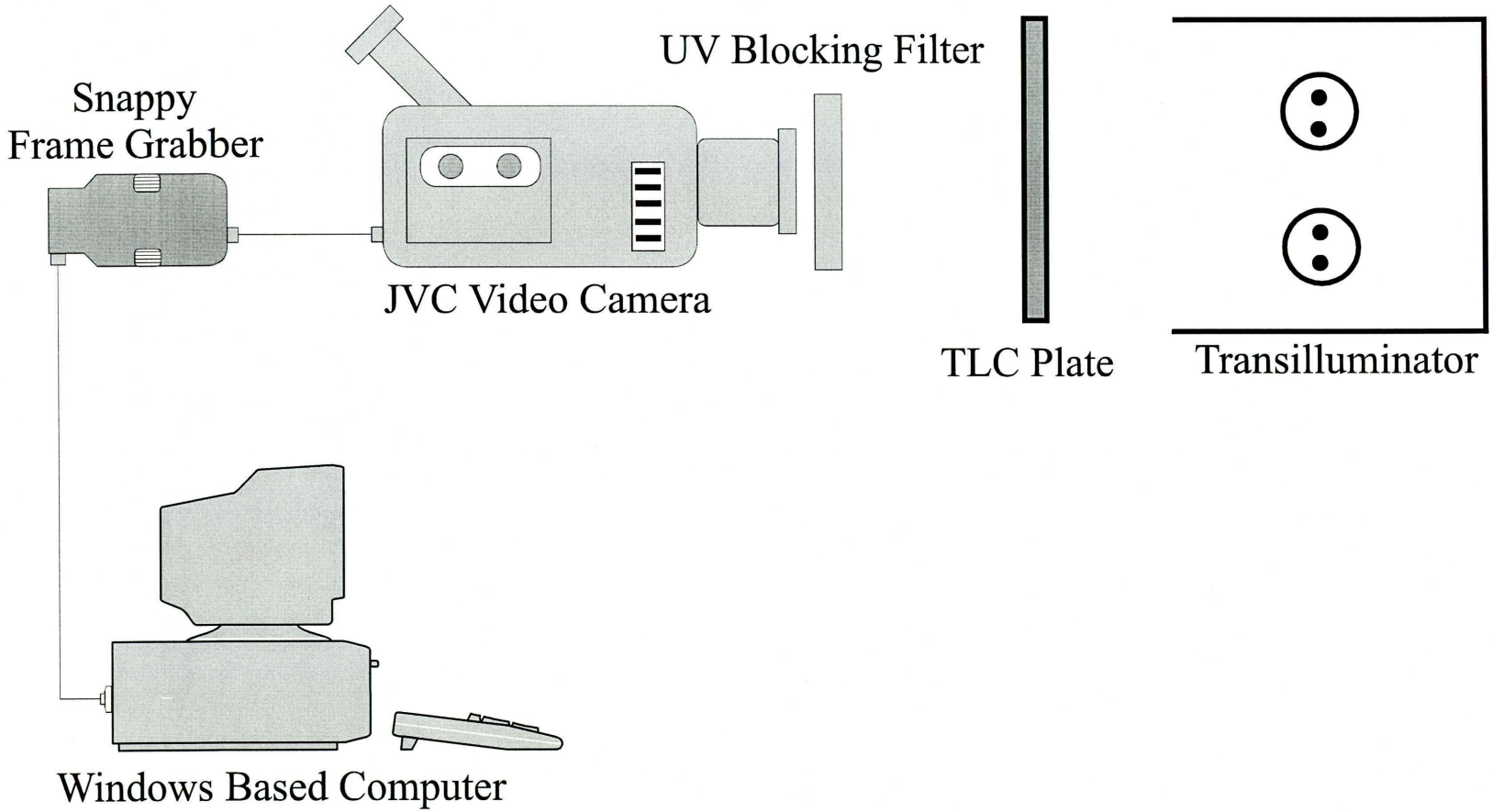


Figure 6.3, Fluorescence detection system configuration

Experimental

Visible Absorbance

The system was configured as described in the previous section. The HPTLC plates were 200 micron thick silica on a 5x5 cm glass backing with the silica side facing the camera. The analyte used to test the visible absorbance was FD&C Blue #1 (Sigma). Varying concentrations of the blue dye were made up with Eppendorf pipettes and applied using the modified sample applicator described in Chapter 2. Application time for the spots was 4 minutes at a flow rate of 2.00 microliters/minute. Regulator pressure was 25 psi and the spots were approximately 1 mm in diameter. The spots were developed with 90:10 ethanol/water and air-dried prior to detection. The illumination source used was ambient room light or a tungsten filament bulb set up in reflection mode, depending on the system being tested. For spectral selection, a red Tiffen filter (Tiffen Red #1) was mounted on the camera lens. The frame grabber obtained images at 320x200 spatial resolution in 8-bit gray scale in highest quality still mode as PCX files. Images were processed using a Labview based program written specifically for the task of converting the PCX format images into 8-bit intensity graphs that can be integrated to provide analytical information. All images were flat-fielded using the plate as the flat field before analyte was applied. The flat fielding process is described in Appendix A.

Fluorescence Quenching

The system configuration for fluorescence quenching is the same as for visible absorption with the following exceptions: The HPTLC plates were purchased factory pre-coated with a fluorescent indicator, designed to fluoresce when illuminated with 254 nm light. Spots were applied in the same manner as described in the visible absorbance section. The filter used for spectral selection was a CVI Laser Corporation LWP-400 filter, designed to reject UV photons. Varying concentrations of oxprenolol (Sigma) were

made up with Eppendorf pipettes and applied to the plate using the modified sample applicator. The plates were developed with hexane/ethanol 90:10 (v/v). Images were in the same format as described in the visible absorbance section. Flat fielding was performed using the plate before analyte was applied as the flat field image

Fluorescence

The system configuration was the same as previously described with the following exceptions: The HPTLC plates were the same plates used in the visible absorbance section. The illumination source is a UV transilluminator, identical to the one described in Chapter 3. The transilluminator was oriented behind the HPTLC plates to illuminate the plate in transmission mode. The spectral selection filter was the same as described in the fluorescence quenching section. The analyte used to test the system was Rhodamine-6-G (Kodak, Laser Grade) applied in the same manner described in the visible absorbance section. The analyte was developed with 100% ethanol and air-dried before analysis. Flat fielding was performed using a Y-48 fluorescent filter described in Chapter 3.

Results

The results from the tests performed were excellent. In each of the three system configurations, linearites and detection limits were obtained, as well as testing system applicability and effectiveness in each situation.

Visible Absorption

The results from the visible absorption curves were excellent, with typical R^2 values ranging from 0.920 to 0.996. Two different illumination sources were used to

determine which source worked best. The two sources were a tungsten bulb desk lamp, commonly used for room lighting. The other source was ambient room light. The results from using these different sources is shown in Figure 6.4. Image analysis time was approximately 15 seconds.

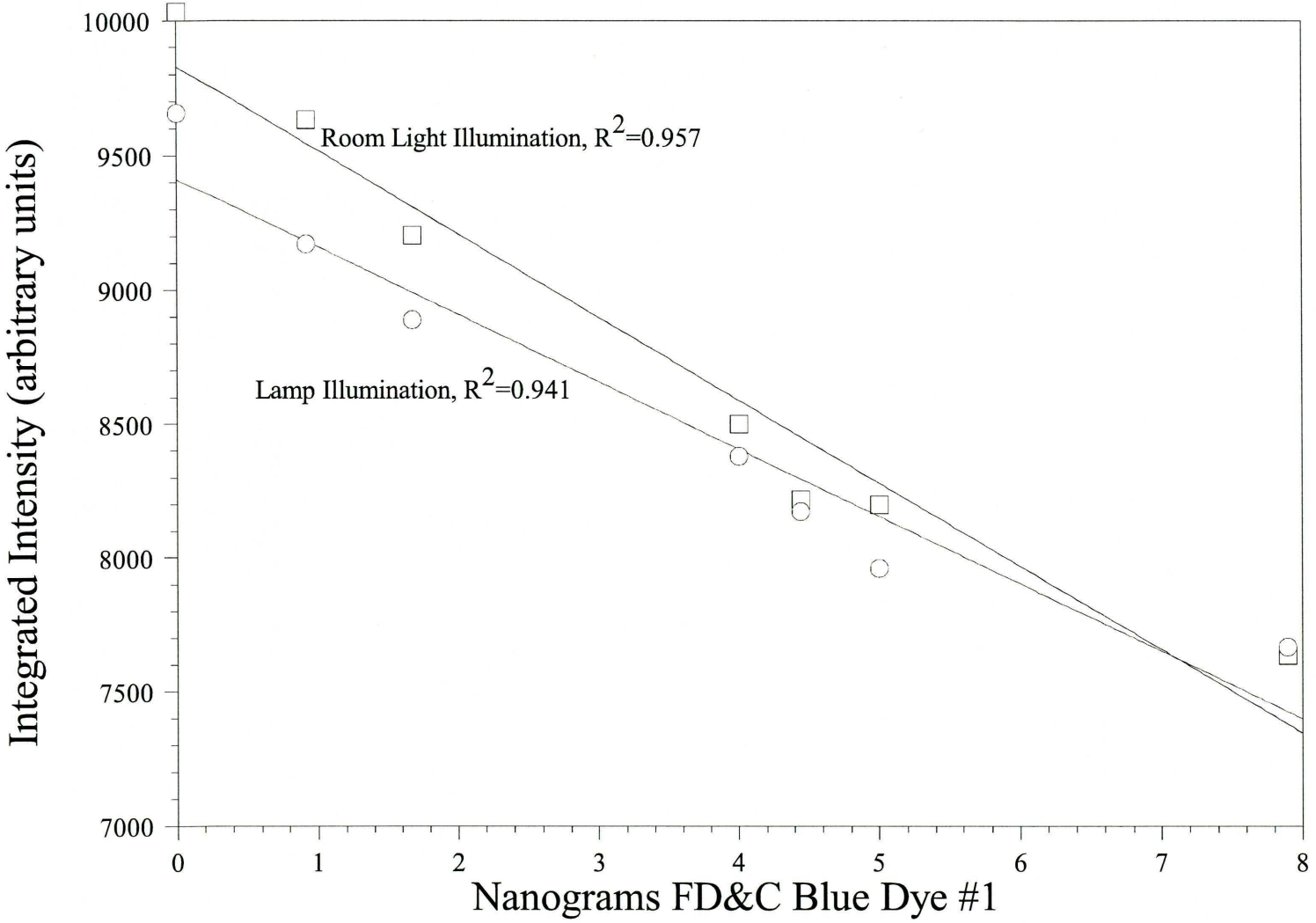


Figure 6.4, Comparison of room light illumination to desk lamp illumination for visible absorbance measurements

As shown in Figure 6.4, the results for using both illumination sources are nearly identical. Relative intensities and linearities are virtually identical, accounting for operator variance in choosing peak areas in each image to integrate. The implication of these results is that a variety of visible illumination sources can be used when operating the system. This is particularly important in applications where a low initial cost is necessary, such as in high school laboratory experiments.

Another experiment performed was the separation and quantitation of a multiple dye mixture. Various FD&C dyes were dissolved in methanol and applied to the plate in the manner previously described. The dyes used were FD&C Red #40, FD&C Yellow # 5, and FD&C Blue dye #1. The dyes were made up in varying concentrations ranging from 60 ng to 1 μg, spotted onto a 5x10 cm Sargent-Welch Octadecyl plastic-backed plate, and developed with phosphate buffer (100 mM, pH 5.5):MeOH 7:3. Various Tiffen filters were used to spectrally select appropriate wavelengths to determine linearity. Flat-fields were taken prior to spots being applied to the plate with the appropriate filter and corrected as previously described. The Tiffen filters used are shown in Table 6-1. The results are shown in Figure 6.5.

FD&C dye	Tiffen filter
FD&C Yellow #5	Dark Blue # 47B
FD&C Red#40	#11 Green 1
FD&C Blue #1	Red #1

Table 6.1, Tiffen filters used with corresponding FD&C dye

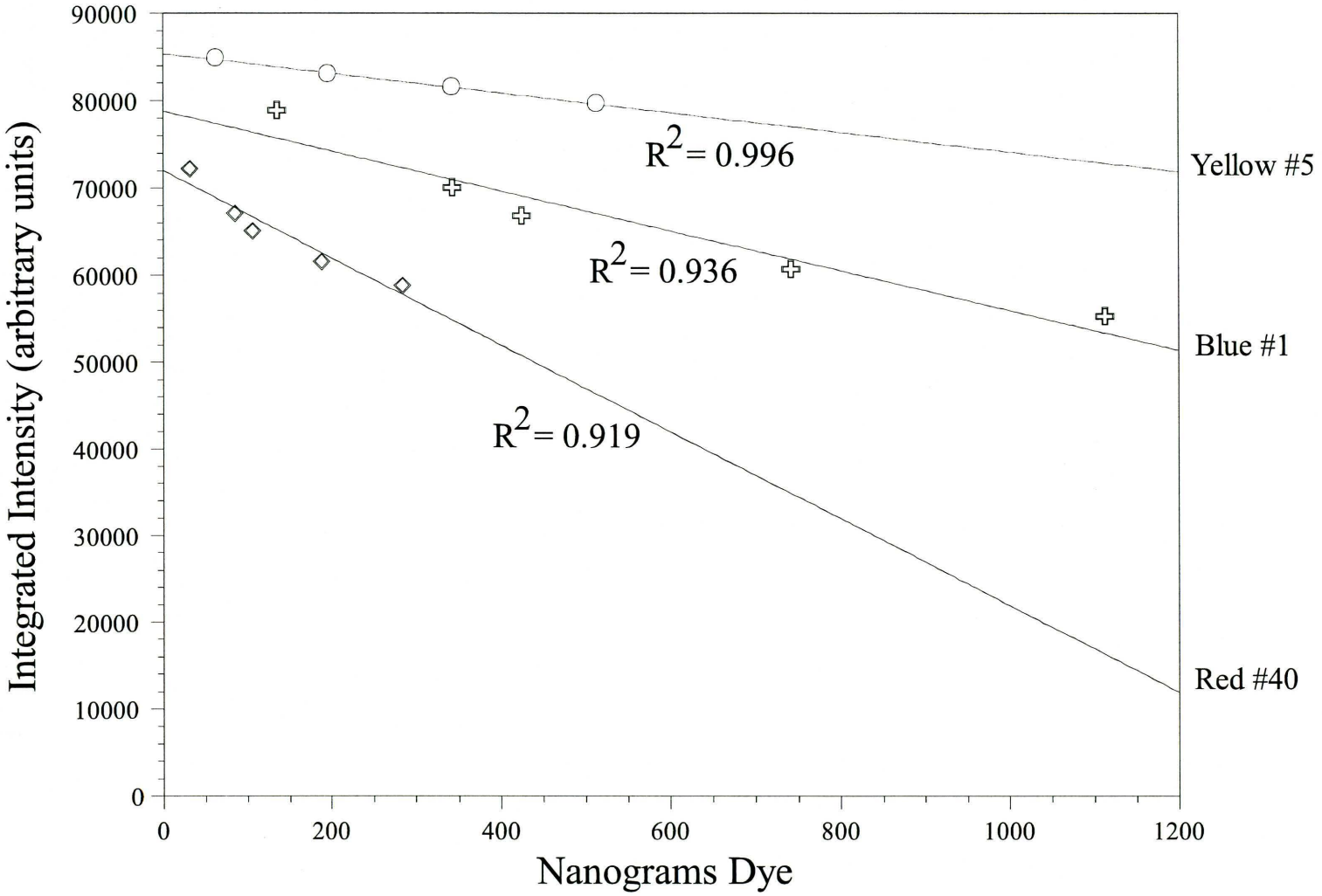


Figure 6.5, Various colored dyes separated on an HPTLC plate detected with video camera and Snappy frame grabber

Again, the results show excellent linearity, with R^2 values typically ranging from 0.920 to 0.993. The video camera-frame grabber combination successfully imaged the plate and returned excellent quantitative results at an extremely low cost. Although the Tiffen filters do not perfectly match the absorbance maximum of the analytes being used, they do provide sufficient spectral selection at extremely low cost, at the expense of slightly reduced sensitivity.

Additional experiments were performed to compare the video camera to the Photometrics PM512, a scientifically operated CCD camera. The PM512 system was set up in reflectance mode with ambient room light illumination. Images from the PM512 were 1 second bias-corrected exposures, and spectrally filtered with a CVI laser filter. Images were then flat-fielded using the plate before analytes were applied, and analyzed with the Labview program. Results from this experiment are shown in Figure 6.6.

The results from the test show, as expected, that the PM512 has better detection limits and linearity, due to the 14-bit Analog-to-Digital converter present in the PM512, as opposed to the 8-bits of digital resolution found in the video camera, but the results from the video camera were still excellent and are more than adequate for a large number of applications.

Fluorescence Quenching

The fluorescent quenching analysis tests, like the visible absorbance tests, were excellent. R^2 values typically ranged from 0.918 to 0.973 and detection limits were on the order of 100 ng. Image analysis time was approximately 15 seconds. The results from performing linearity tests are shown in Figure 6.7. Again, as expected, the PM512 showed better linearity and detection limits, but the results from the video camera were still excellent, and more than adequate for many separations.

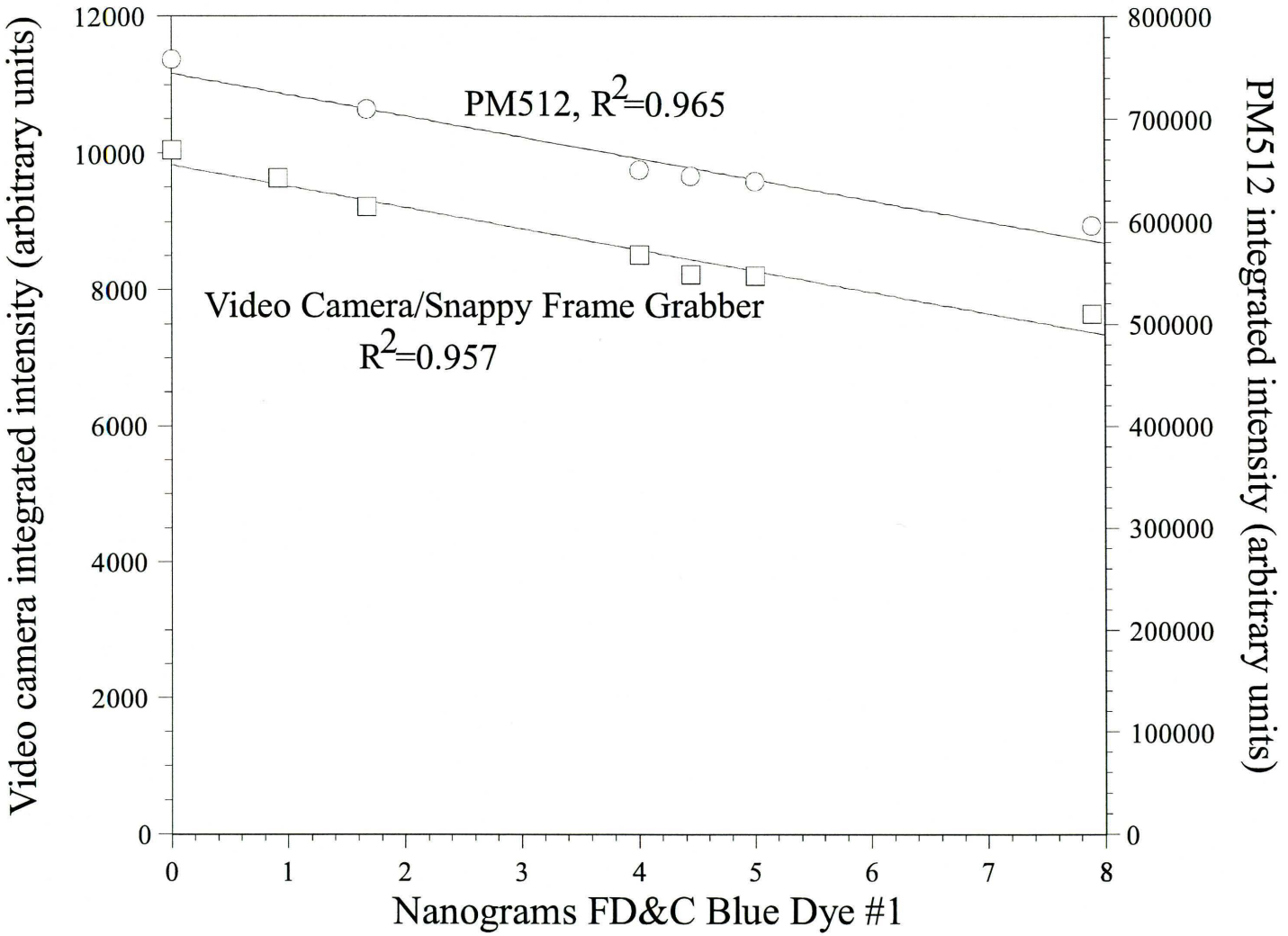


Figure 6.6, Comparison of video camera and PM512
for visible absorbance

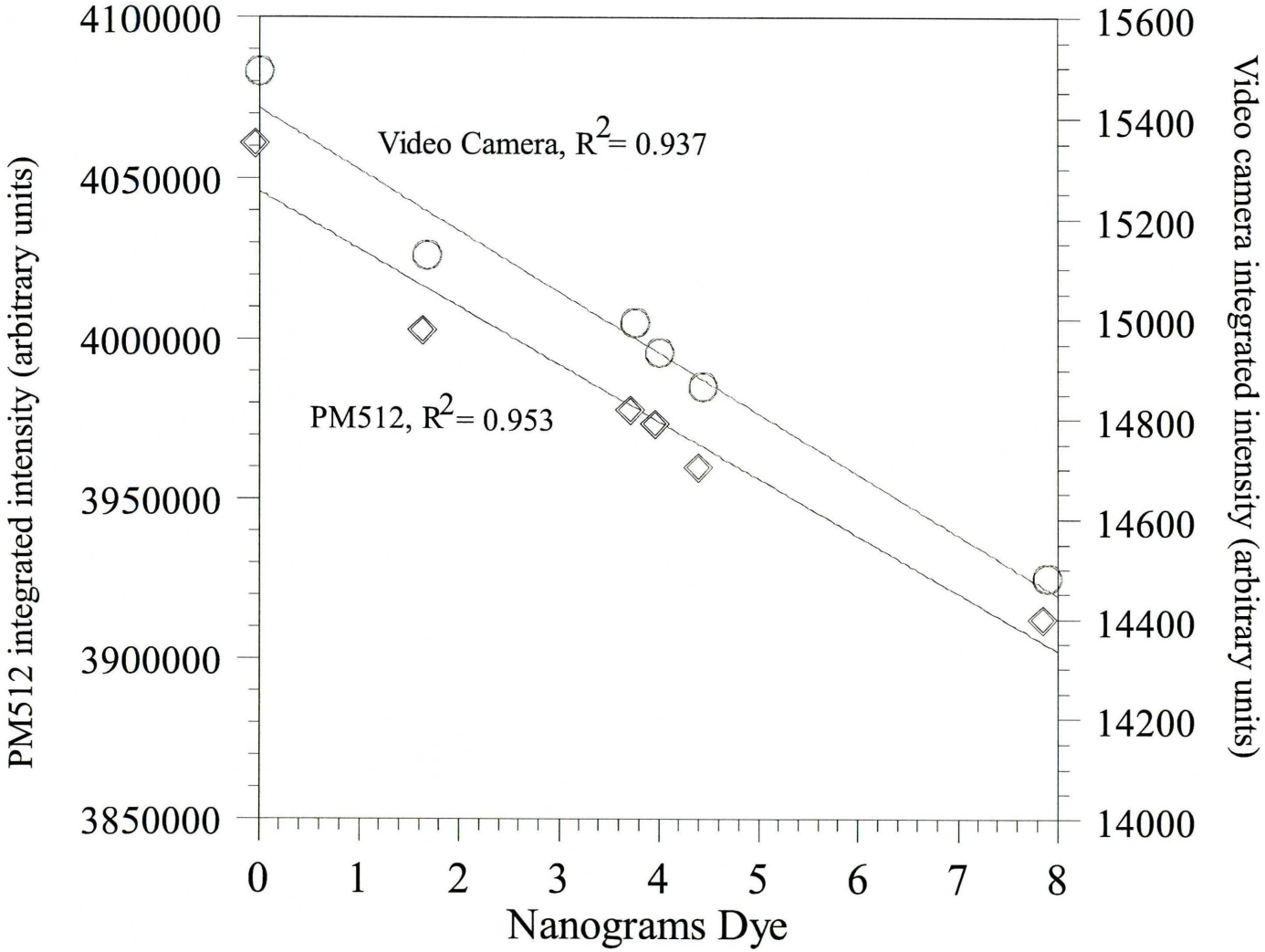


Figure 6.7, Comparison of PM512 to video camera for fluorescent quenching

Fluorescence

The ability of the system to perform fluorescent analysis was tested using Rhodamine-6-G (R-6-G). Results are shown in Figure 6.8. Linearities were excellent, on the order of 0.965, with detection limits approximately 3 ng. Comparisons were also performed between the video camera and the PM512 camera. Images of the same plate used for video camera analysis were used to compare the two systems. A UV transilluminator was used to illuminate the analyte in transmission mode. Images from the PM512 were 5 second exposures, flat-fielded with the Y-48 filter, and integrated with the Labview program. Results are shown in Figure 6.8.

Again, as expected, the PM512 shows better linearity and sensitivity than the video camera, but the video camera still performs the job adequately for many situations. However, fluorescent analysis of low concentrations (sub ng) pose somewhat of a problem for the system. Since the integration time is not adjustable, the $1/30^{th}$ second integration time is somewhat unsuited to fluorescent analysis where longer integration times are required. This limits to a certain degree the field of analysis that the system is applicable to, since it cannot detect lower concentrations of the analyte of interest. It is in these cases where the use of a scientifically operated CCD is necessary to perform the analysis of interest, since the integration time is adjustable and greater intensities can be obtained on the chip, and lower detection limits and better sensitivity obtainable. Use of the video camera-frame grabber for fluorescent analysis is best suited to higher concentrations of analyte.

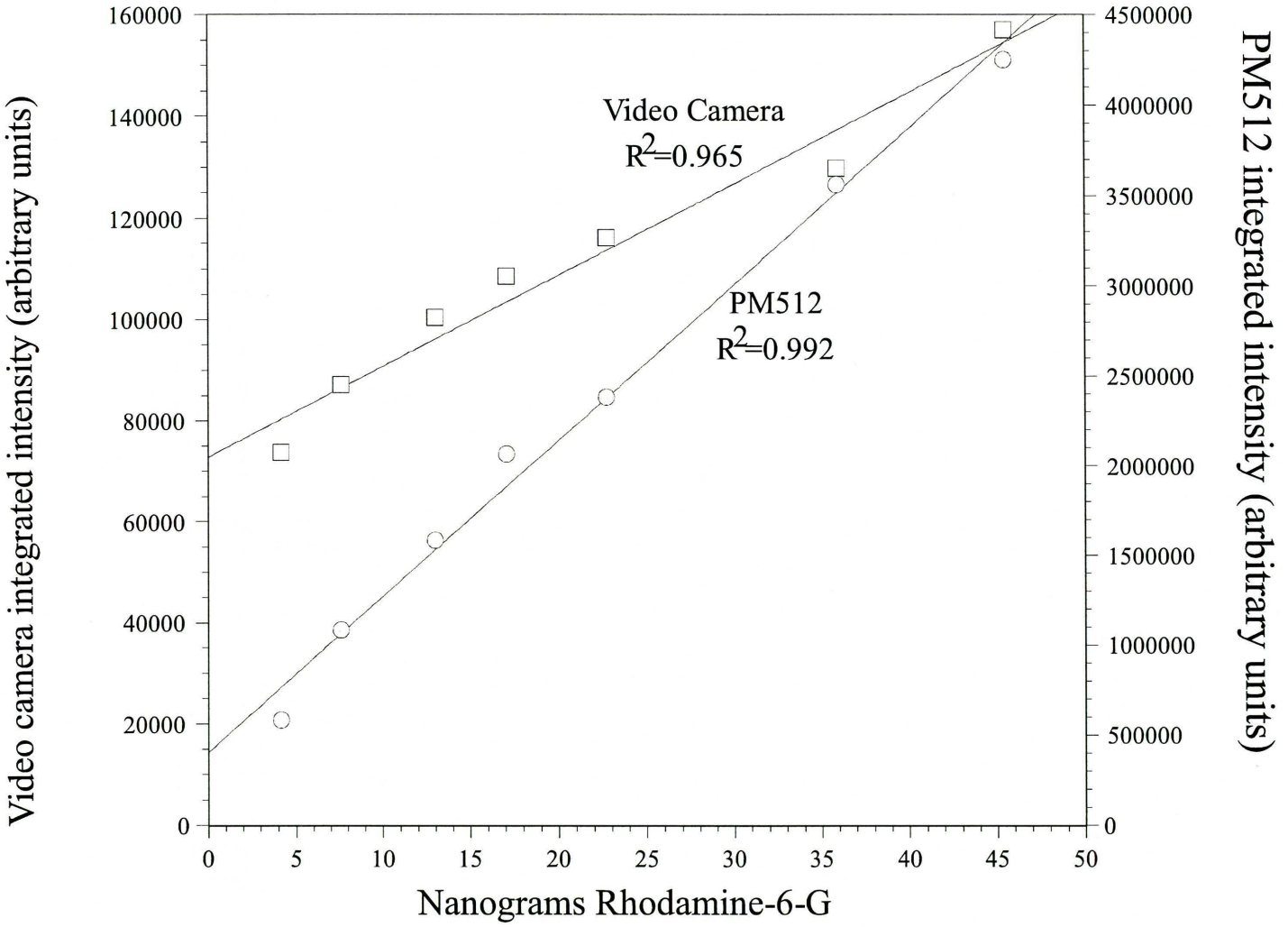


Figure 6.8, System comparisons between the PM512
and video camera/frame grabber system for fluorescent detection

Additional Experiments

Additional experiments were performed to test the reproducibility of the system in obtaining images of the same plate repeatedly. Ten different images of the same plate with no spots were taken over the course of approximately 5 minutes to test the reproducibility of the image falling on the chip. The system configuration for this test was fluorescent quenching mode. Each image was captured in "highest quality still" mode and in each image, the same coordinates in each image were used to sum intensities. The results show a variance of 1.6% between the images. The same test was also performed in the visible with a variance of 1.3%. This variance can be ascribed to two factors, the inherent reproducibility of the camera as well as source fluctuation during the measurements. Either way, the reproducibility of the images is more than good enough for quantitative analysis. An additional test was performed to test the reproducibility of the camera when multiple images of the same spots were taken after the plate was removed from the plate holder and repositioned before each measurement. The system setup was the same as previous fluorescent measurements. The results show a 0.93% variance in spot intensity, which, like the fluorescent quenching reproducibility experiment, can either be attributed to inherent system reproducibility or source drift. Again, the results obtained are more than good enough for quantitative analysis.

System Tests

Quantitative Determination of Food Dyes in M&M Candies

To determine the applicability of the system to an educational setting, such as a high school or college-level lab, a separation was performed that would illustrate the quantitative ability of the system on a system familiar to the student. This system chosen was the separation of FD&C dyes present on M&M brand candies. (Mars Inc. Hackettstown, NJ) The analytes to be separated are familiar to most students and are

brightly colored in the visible, helping to illustrate the principles of separation and detection involved. The separation itself is taken from a HPTLC demonstration kit manufactured by Sargent-Welch. (Sargent-Welch Scientific Company, Chromatography Instruction Kit, Part # S-18930-02). The experimental portion of the separation is relatively simple to perform. The dyes of interest are various FD&C dyes (FD&C Blue #1, Red #40, Red #3, and Yellow #5), readily soluble in water. M&M's of varying colors are quantitatively dissolved in 2.00 mls water until the dye or dyes of interest are completely dissolved. This can be noted by the disappearance of the color of the M&M and the appearance of the white layer beneath the dye. The solution corresponding to each color of M&M is spotted onto a 5x10 cm Sargent-Welch C-18 plate. Standards containing all four dyes in concentrations covering the range of concentrations found on the M&M's were applied on the same plate to create a calibration curve. For this application, the sample was applied using the modified sample applicator, but Drummond micropipets, described in Chapter 2 will also perform the application process adequately. The plates were developed approximately 5 cm with phosphate buffer (100 mM, pH 5.5) / MeOH 7:3. Plates were imaged using ambient room light as illumination and various Tiffen filters were used for spectral selection. Tiffen filters, chosen for their low cost and large availability, were used with the dyes listed previously in Table 6.1.
Images of the plate in "highest quality still" mode were taken at 320x200 resolution. The images were saved as PCX format files and integrated.

The results from the experiment shows that the video camera performs adequately the task of quantitation of the dyes of interest in the visible portion of the spectrum, as well as demonstrating the ability of HPTLC to be a quantitative technique. Figure 6.9 shows the results obtained from generating a calibration curve to determine the dye amounts.

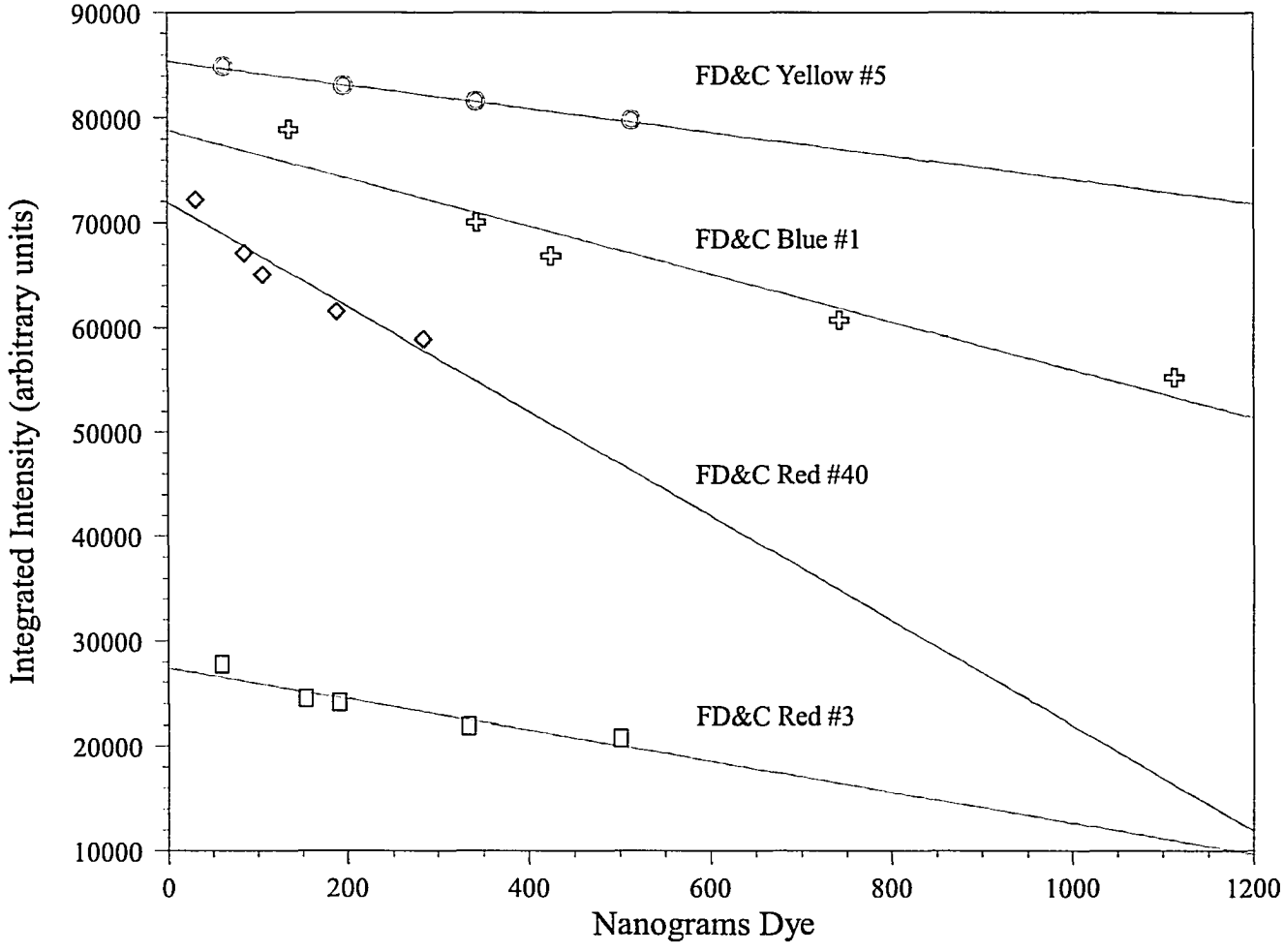


Figure 6.9, Calibration curves generated for the determination of dyes in M&M Brand Candies

The results show the utility of using the video camera to image the plate. Results were obtained in a very short time, and at a very low cost, and the principles of quantitative determination of the dyes are easily demonstrated. As a whole, the experiment not only instructs on the principles of chromatographic separation, but also instructs on the quantitative determination of the results obtained from the separation. Use of the video camera is applicable to a wide variety of chromatographic separations that involve colored compounds, and could easily be modified to almost any pre-existing experiment where quantitative information is desired. Slight experimental variations from the described procedure are necessary, however, in implementing this experiment in an educational setting, namely the use of microcaps for quantitative sample application. This experiment is an excellent example of the low cost, high throughput abilities of low cost quantitative HPTLC and could easily be incorporated into laboratory experiments ranging from high school through college-level courses.

Quantitative Analyses of Analgesic Tablets

To further test the abilities of the system for quantitative determinations in an educational setting, a slightly more involved experiment was performed. Analgesic analysis of commercial tablets represents a more advanced system to test the quantitative abilities of the system under different conditions. The analgesic analysis involves the quantitative determination of three components present in commercial pain-relief products, aspirin, acetaminophen, and caffeine. The experiment involves obtaining different brands of analgesic that contains one or more of these compounds. In this case, the brands Excedrin, containing all three components, Bayer Aspirin, containing aspirin, and Extra-Strength Tylenol, containing acetaminophen, were chosen to conduct the experiment. The procedure was obtained from Sherma and Rolfe.(6.1) Aspirin, caffeine,

and acetaminophen were obtained from Sigma and solutions were made in concentrations bracketing expected concentrations to be found in the tablets. Methanol was used as the solvent. Tablets of the commercial analgesics were crushed and quantitatively transferred to volumetric glassware and dissolved with MeOH. The 5x5 glass-backed F254 HPTLC plates were spotted with standards and unknowns onto the plate. These solutions were spotted with the modified sample applicator at 2 μL/minute for 4 minutes, with spot sizes approximately 1.5 mm. Figure 6.10 shows a sample image and results from the separation are shown in Figure 6-11.

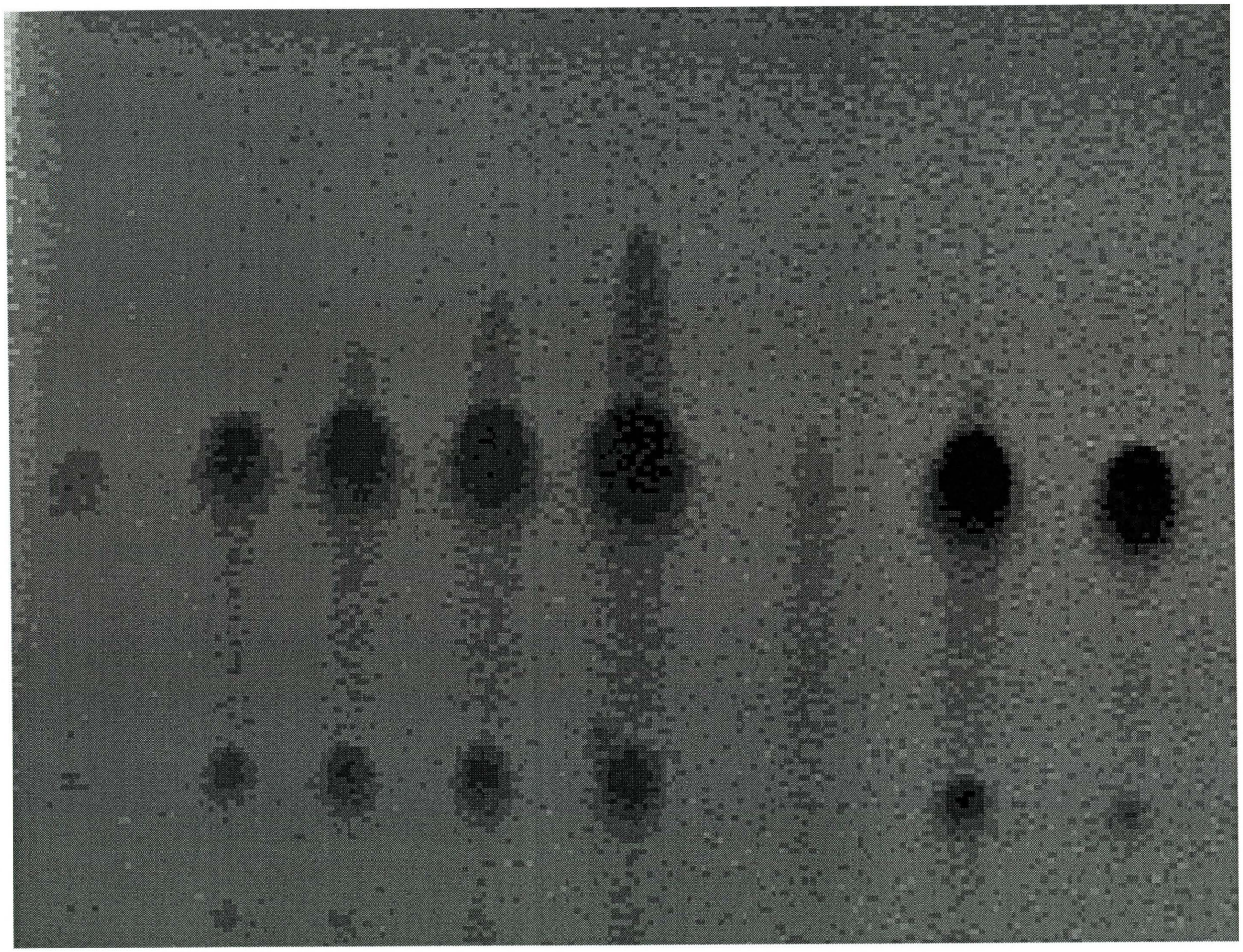


Figure 6.10, Image of analgesics obtained with video camera-frame grabber system

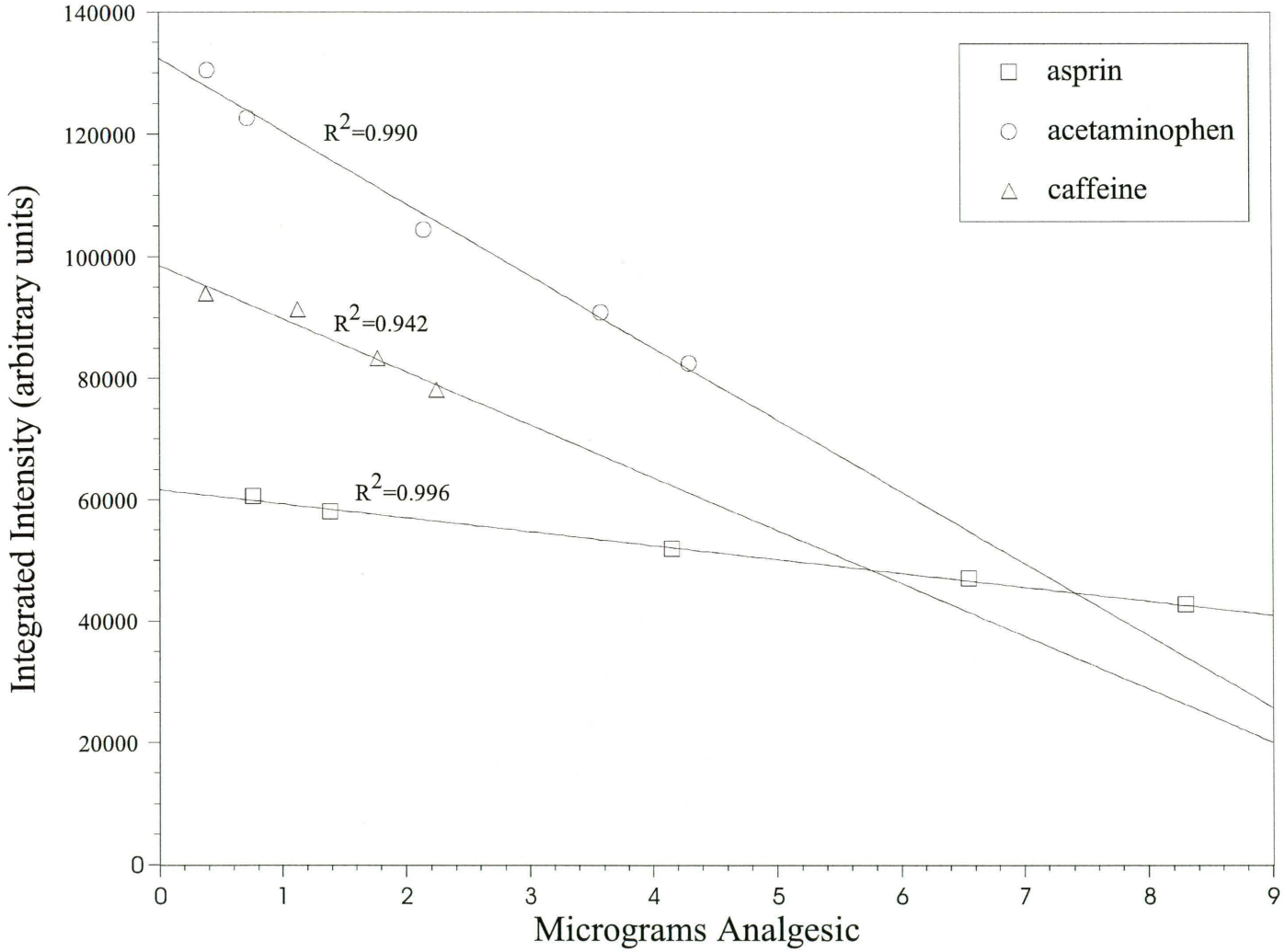


Figure 6.11, Analgesic analysis calibration curves using video camera and Snappy frame grabber

The calibration curves from the separation were excellent. Linearities and detection limits are shown in Table 6.2.

Analyte	Detection Limit	Linearity
Acetaminophen	50 ng.	0.992
Aspirin	300 ng.	0.988
Caffeine	200 ng.	0.925

Table 6.2, Detection limits and linearities obtained for analgesics imaged with video camera-frame grabber

The results from the calibration curves show good linearity, but the expected results of the tablet analysis of aspirin and caffeine were considerably off from manufacturer quoted values. This is likely due to the extraction procedure rather than the video camera itself. The problem with aspirin is that it gives abnormally high values when extracted from a tablet rather than made from pure solution. This may be due to a co-migrating species that interferes with good quantitation and makes the value appear unusually high. Caffeine results can vary and give good results some days and not others. This may be due to the properties of the caffeine itself, since caffeine has a tendency to sublimate when exposed to air. This could cause lower than expected results depending on how long the analysis procedure took. The acetaminophen results were consistently good, with errors at usually around 5 to 10%. This is about the value quoted by the authors of the method. Overall, the video camera performed well, but the extraction procedure for the tablet analgesics needs further method development to ensure accurate representations of the tablets are spotted onto the plate, and that possible co-migrating species do not interfere with quantitation of the analytes of interest.

Quantitative Analysis of Oxprenolol

To test the system in comparison to a commercial instrument, an application was chosen that relied on scanning densitometry for quantitative analysis. The application is the identification and determination of oxprenolol, a beta-blocking drug used to treat hypertonicity in blood. Cases of suicide have been reported where concentrations in blood up to 60 PPM are found.(6.2) In extracts from urine, oxprenolol occurs mainly as its metabolized product, but sufficient amounts of non-metabolized form are excreted to allow its safe identification.(6.3) The primary advantages to performing the separation using HPTLC is the high sample throughput at low operating cost. The system comes from an application developed by CAMAG.(6.4) The CAMAG system uses a scanning densitometer to quantitatively determine oxprenolol levels extracted from blood. Application requirements are mid-nanogram detection ranges performed on F254 HPTLC plates. In this study, calibration curves corresponding to those given in the application note were prepared, although no real-world samples were performed, due to the lack of test subjects. The response of the video camera to the levels of oxprenolol on the plate were used to determine the applicability to a system designed for a scanning densitometer. Samples were prepared by quantitatively dissolving oxprenolol (Sigma) in MeOH and using this as a stock solution to make up the remaining solutions to be spotted and analyzed. Spots were applied using the modified spotter described in Chapter 2. Spots were applied for 4 minutes at 2 μL/minute and were approximately 1.5 mm in diameter. The system was set up in fluorescence quenching mode and frames were grabbed at 320x200 resolution in "highest quality still" mode. The illumination source was the mercury pen lamp used in the previous fluorescence quenching experiment. The results from the calibration curve is shown in Figure 6.12.

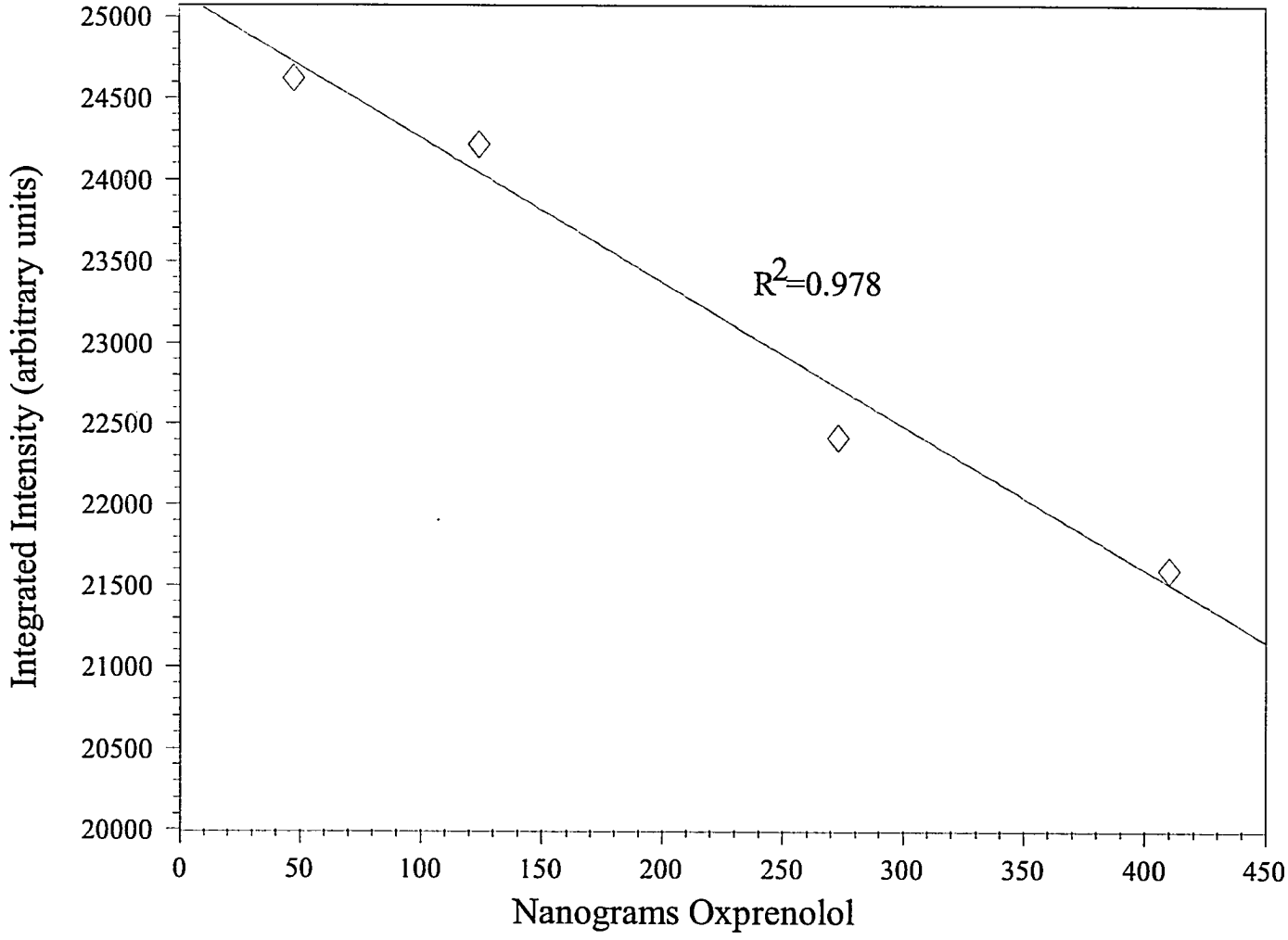


Figure 6.12, Fluorescence quenching analysis of oxprenolol using video camera and Snappy frame grabber

The results show that the video camera is at least as good as a scanning densitometer for detecting the analyte on the plate. The detection limits from using the video camera were approximately 50 ng. The application note from which the procedure was obtained quotes a detection limit of below 50 ng, but no precise number is given. It is clear, however, that the video camera-frame grabber combination performed the job of detecting the analyte of interest, oxprenolol, at the same level of concentration that the scanning densitometer does, and at significantly less cost, significantly shorter analysis time, and significantly simplified ease of use. This preliminary data shows the utility of the use of the video camera as a replacement for the scanning densitometer. The scanning densitometer and the video camera-frame grabber combination both performed the separation and quantitation of the analyte of interest to adequate levels, but the video camera-frame grabber unit costs on the order of $600 to purchase the equipment necessary to quantitate the analyte, whereas the scanning densitometer costs approximately $20,000 dollars to obtain the same analytical data. Additionally, the video camera-frame grabber system has the added benefit of array detection, which shortens the analysis time from approximately 30 minutes for the scanning densitometer to about 3 minutes total analysis time. This in addition to the ease of use of the video camera-frame grabber system as compared to a scanning densitometer makes the use of the system extremely attractive as a replacement to the scanning densitometer in many applications.

Discussion

The previous examples show that the video camera-frame grabber detection system performs well a wide variety of separation and detection applications. The ability to use the system in fluorescence, fluorescence quenching, and visible absorbance makes it applicable to many separation techniques that currently use scanning densitometry as a

quantitation technique. Additionally, the extremely low cost and ease of operation of the system make it applicable in both an educational atmosphere as well as a scientific setting. The system operates on roughly the same performance level as a scanning densitometer, but at an order of magnitude decrease in cost and an additional order of magnitude decrease in analysis time and at considerably simplified ease of use. These features make the video camera-frame grabber system an extremely attractive alternative to the use of scanning densitometry, applicable in a wide variety of fields, from an educational setting, to an analytical laboratory.

CHAPTER 7

CONCLUSIONS AND IMPLICATIONS

As shown in the preceding chapters, the combination of array detection and high performance thin layer chromatography are a powerful means of detecting and quantitating a large number of analytes in a short amount of time. The benefits of HPTLC, including low cost per separation, high throughput, good separation ability, easy sample preparation, static detection, and total sample accountability, combined with the benefits of array detection, including reduced analysis time, improved precision, high spatial resolution, and low detection limits, make an extremely powerful separation and quantitation system that is applicable in a wide variety of fields. The advantages of each of the preceding chapters is summarized in Table 7.1.

Area	Results	Advantages over existing technology
Nebulization Sample Application	• High precision • High accuracy • No contact with separation media • Semi-dry sample application	Lower cost and higher performance than current application methods
Array Detection for Aflatoxin Analysis by HPTLC	• Improved throughput • Real-time monitoring • Easier operation • Significantly reduced analysis time	Ability to sample more thoroughly allows for more accurate determination of potentially dangerous levels of aflatoxins
Array Detection for Amino Acid Analysis by HPTLC	• Improved throughput • Easier operation • Significantly reduced analysis time • Problem of analyte decay eliminated	Improved throughput and significantly reduced analysis time over current methodologies allows for a more accurate determination of free amino acid content
Near Infrared Array Analysis of HPTLC plates	• Qualitative and quantitative information available • Rapid detector response • Multiple detection modes • Rapid analysis possible	System allows for qualitative and quantitative analysis of a very large number of components on a HPTLC plate in a very short time
Low Cost Array Detection for HPTLC	• Low cost array detection • Simple operation • Multiple detection modes • Good linearity • Low detection limits	Array detection for HPTLC becomes extremely low cost and simple to operate with performance levels comparable to existing technology

Table 7.1, Summary of advantages of each system studied

APPENDIX A

FLAT FIELD AND BIAS CORRECTION

In an ideal detector, every detector element would respond equally to equal excitation. In reality, each detector element in the CCD has a different offset, dark current, and slope. The purpose of bias correction and flat field correction is to correct to some degree the differences encountered in each individual detector element. To do this, the offset and slope of each individual element must be determined. This process requires at least 2 calibration images to be acquired, a bias image and a flat field image. The bias image corrects for additive effects and the flat field corrects for multiplicative effects.

Bias Correction

The bias image attempts to correct for the bias or offset of the detector system output at zero signal levels. The offset , which may not be equal for all detector elements, is removed by subtracting off an image obtained with no light falling on the detector. The bias image is read out as quickly as possible to avoid dark current accumulation during the read out process. Typically, a bias image is subtracted from every image taken, whether flat fielding is performed or not.

Flat Fielding

To obtain an image of a planar surface, it is necessary to have an illumination source. To obtain analytical information about analytes imaged on a HPTLC plate, it is necessary to either have a uniform illumination across the surface of the plate, or have a process for correcting for uneven illumination so that the analytical information obtained is valid. To illuminate the entire surface of the HPTLC plate evenly is expensive and

requires a relatively complex optical arrangement. The alternative to this is to employ a mathematical means for correcting uneven illumination to obtain a corrected image that contains valid analytical information. This process is known as flat fielding. Flat fielding corrects for spatial fluctuations in source intensity and fixed pattern variations in detection sensitivity related to optical components or the detector itself. The process used to correct all images obtained in this work is similar to the process described by Sutherland and co-workers.(A.1) In essence, a map is needed of spatial illumination that occurs across the plate. Once this map is obtained, the image of the plate with the analytes present can be corrected for the amount of illumination that is present at each location on the plate. The final image obtained once the flat fielding process is obtained is an excellent approximation of even illumination of the entire plate. There are several ways to correct for uneven illumination across the plate, depending on the way that the plate itself is being imaged.

All flat fielding is generally done in the same way. Division is used to correct the final image. HPTLC plate images are corrected as described in equation 1.

$$\text{Corrected Image} = \frac{\text{Adj.Orig.Image}}{\text{Adj.FieldImage}} (\text{Adj.FieldImage})_{\text{average}} \quad (1)$$

where,

$$\text{Adjusted Original Image} = \text{Original Image} - \text{Bias Image} \quad (2)$$

$$\text{Adjusted Field Image} = \text{Field Image} - \text{Bias Image} \quad (3)$$

As described in equations 2 and 3, both the field image and the original image of the HPTLC plate are corrected for any fixed pattern and electronic offsets with the subtraction of a bias image prior to field correction of the plate image.

Essentially, each pixel in the original image (HPTLC plate) is divided by the equivalent pixel in the field image. The result of the division for each pixel is multiplied by the average value of pixel intensity in the field image to return intensity values back to near the values of the original image. The final image obtained is corrected for uneven source illumination and useful analytical information can be extracted from the image.

Examples

Fluorescence requires a separate fluorescent filter be used to map spatial differences in ultraviolet illumination from the source, typically, a fluorescing filter that is excited at the same wavelength as the analyte of interest. The filter is spatially very uniform in its fluorescent properties so that it fluoresces in direct proportion to the amount of UV light striking the filter. This is an excellent correction method for mapping out UV spatial source distribution. The disadvantage to the use of the filter is that only UV spatial differences are mapped. HPTLC plate inhomogeneity is not accounted for and such factors as silica thickness and localized fluorescent impurities are not corrected. The plate itself can be used to obtain an image prior to the analytes being applied, but long exposures are required and the technique is only partially effective. Much of the image intensity is accumulated dark current, but localized impurities and plate inhomogeneities can be somewhat accounted for in the final image.

Another method for flat field correction when fluorescent quenching analysis is being used is to use the precoated fluorescent plates for the flat field image before any analyte is applied. Since the plates are coated with the fluorescent dye, they fluoresce

green when excited by 254 nm light, allowing it to be seen by the camera. This allows a "before-and-after" image to be taken. This accounts for all inhomogeneities present in the plate to be corrected. Such factors as silica thickness, fluorescent coating inhomogeneities, localized impurities, as well as uneven UV illumination are accounted for and corrected. This method was chosen for the precision study discussed in Chapter 2 because it eliminates more sources of error than any other method of flat fielding. Figure A.1 shows the effectiveness of each flat field correction method in correcting such factors as plate inhomogeneity, spatial source drift, and temporal source drift. Figure A.1 also shows the quality of the results obtained in accuracy and precision calculations of spot intensity. The best method for flat field correction is the use of the precoated fluorescent plates as a flat field image, since individual plate differences are corrected for each different plate. Essentially, the "before-and-after" images of the plate provide the best flat field correction available. The only disadvantage to the use of this method is the fact that a fluorescence quenching media must be used for the separation, and this is not always applicable to a particular separation. Overall, the flat field process is an excellent method for correction of obtained images to obtain valid analytical data.

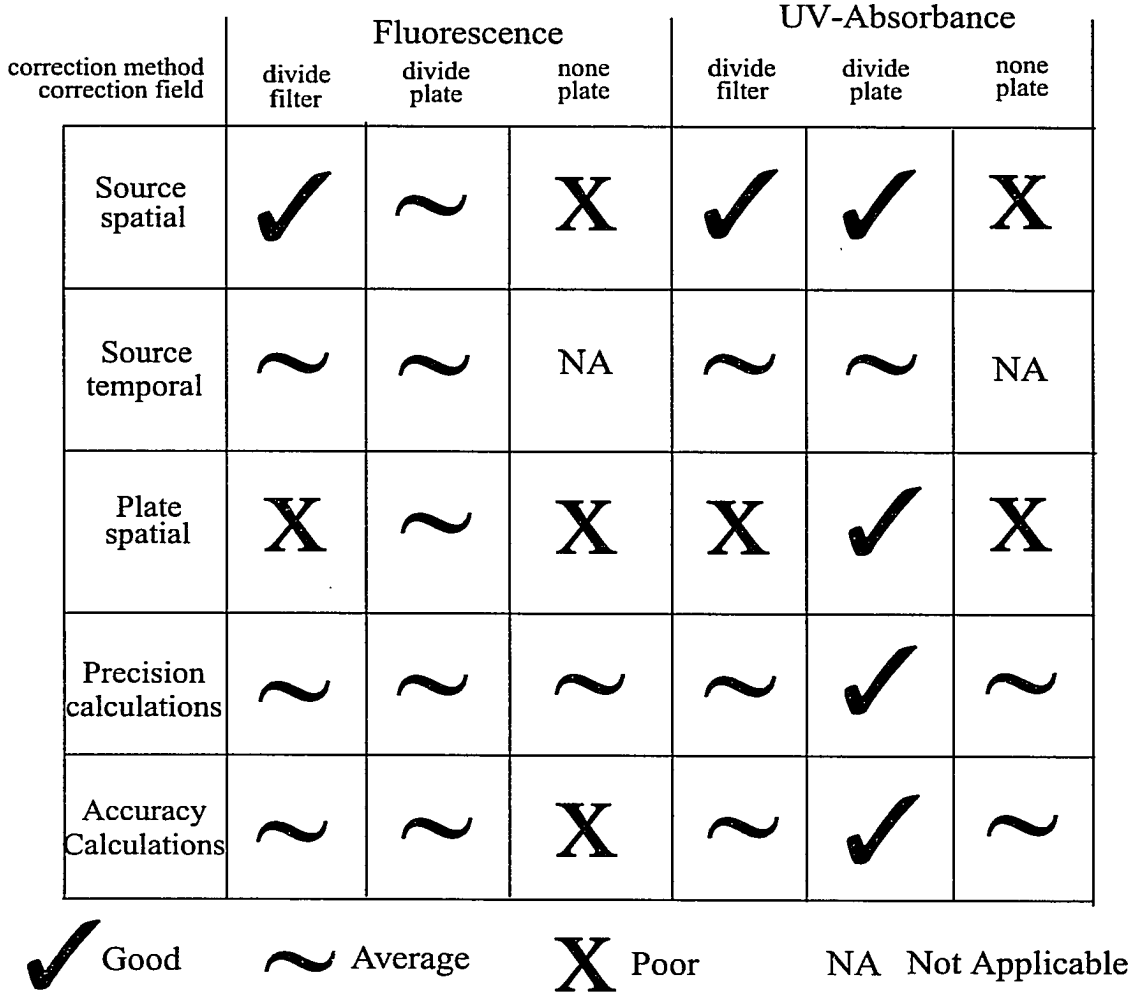


Figure A.1, Flat field options

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Appendix A

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