# Thin-Layer Chromatography for Binding Media Analysis

SCIENTIFIC

TOOLS

FOR CONSERVATION

Mary F. Striegel

Jo Hill

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experts in related fields. Future volumes will be devoted to the use of infrared
spectroscopy, photography for conservation, inert atmospheres, and microscopy.

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## Foreword

Among the various methods for the scientific examination of works of art, thin-layer chromatography is a unique and useful tool. It is a simple and fast method for visual assessment of a wide variety of substances. Most recently, the ability to provide quantitative results has significantly improved its use and application. The need for a larger sample size than that required for other methods is offset by the speed with which the analysis can be undertaken.

This publication is the outcome of research methods and techniques developed at the Getty Conservation Institute in recent years. The research was used as the basis for a successful course taught at the Institute aimed at making the technique more accessible and better known by conservators and conservation scientists.

Since its inception, the Getty Conservation Institute has sought ways of making accessible to practicing conservators scientific research methods that bridge the gap between high-level technology and everyday practice. By offering a step-by-step approach; detailed descriptions of techniques, analyses, and interpretations of results; and a selection of protocols, the authors of this publication have contributed significantly to that end. They bring to the conservator's studio knowledge and research findings amassed in the laboratory over the course of several years.

This is the first of the Getty Conservation Institute's Scientific Tools for Conservation series, publications designed to provide practical procedures and methodologies in the field of conservation.

We are particularly grateful to Mary Striegel and Jo Hill for their dedication and effort in the development of this research, Dusan Stulik for his unhampered enthusiasm and guidance in this area, Dinah Berland for coordinating this publication, Anita Keys for production coordination, Nomi Kleinmuntz for editing the copy, and Garland Kirkpatrick for design.

Comments from our readers will help improve future publications and assist the Institute in directing its efforts toward areas of benefit to the conservation profession and related fields.

*Miguel Angel Corzo*
*Director, The Getty Conservation Institute*

# Preface

This book is the result of research and development efforts for "Methods in Scientific Examination of Works of Art: Thin-Layer Chromatography," a course held at the Getty Conservation Institute from 28 February to 4 March 1994. The course familiarized conservators and conservation scientists with thin-layer chromatography (TLC) as a method of binding media analysis. This course was organized jointly by the Training and Scientific Programs of the Getty Conservation Institute and was the second workshop in the series titled "Methods in Scientific Examination of Works of Art."

The theory, practical techniques, and standard operating procedures for thin-layer chromatography as applied to conservation problems are detailed in this publication. It is divided into two parts: the handbook and the protocols. The handbook serves as a primer for the basic application of thin-layer chromatography to the analysis of binding media, adhesives, and coatings found on cultural artifacts. In the second part, the protocols provide step-by-step instructions for the laboratory procedures involved in typical analyses.

The authors wish to thank the team who assisted in the endeavors associated with the course and this publication. They include Marta de la Torre, Michele Derrick, Valerie Dorge, Henry Florsheim, Melena Gergen, Cecily Grzywacz, David Nurok, Andrew Parker, Michael Schilling, Dusan Stulik, Arie Wallert, and Blanca Zimmerman. We would like to thank the conservators and institutions who provided samples detailed in this report, including Leslie Bone, M. H. de Young Museum; Eugenia Ordonez, Museum of Modern Art; and Brian Considine and Jerry Podany, J. Paul Getty Museum.

We also wish to thank Chandra Reedy for the Introduction to this publication.

*Mary F. Striegel*
*Jo Hill*

# Introduction

In the study and conservation of art and artifacts, natural organic materials are frequently encountered in components such as coatings, binders, and adhesives. The identification of these materials is often crucial in the effort to characterize the technologies employed by artists and craftspeople, to understand deterioration processes and causes, or to plan an appropriate conservation treatment. Yet, many institutional and private conservation laboratories have restricted analytical facilities, personnel, and budgets, putting many analysis techniques beyond their reach. Thin-layer chromatography (TLC) can help fill this gap.

TLC is used to separate components in a mixture and to identify unknown materials by comparing their separation pattern to that of known reference materials. It has been used to identify a wide range of materials relevant to art and artifacts. TLC holds advantages over many alternative analytical techniques in that it is relatively simple, rapid, and inexpensive to perform. The practicality of setting up facilities to conduct TLC means that it is within the reach of essentially any laboratory, unlike many other analytical methods. Thus, wider dissemination of practical information about TLC can greatly affect the type and amount of research that can be done with art and archaeological objects.

This book derives from materials originally prepared for a course on TLC for conservators that was offered by the Getty Conservation Institute in 1994, with Mary Striegel as the principal instructor. The information is therefore presented in a didactic manner. The book gives the theoretical background of the technique, along with practical information about how to apply TLC to art materials. The text is clearly written and logically organized and comprises a well-rounded presentation.

The authors stress that TLC is not new to conservation. They review the basic theory of the technique and its place among other chromatographic techniques. They also briefly summarize past applications of TLC to the analysis of art materials. However, one purpose of this publication is to update the conservation field on recent advances in TLC that are pertinent to the analysis of relevant materials, such as binding media, varnishes, and adhesives. Thus, there is an emphasis on new approaches that have appeared in the analytical chemistry literature in recent years. Information from that literature has been extracted for this publication and discussed in the context of its application to art materials.

Another purpose of this book is to provide practical information in a format accessible to conservators and other nonchemists interested in learning how to perform TLC themselves. One way in which the book fulfills this function is to present a series of protocols that serve as step-by-step guides for conducting TLC. Protocols for the identification of proteins, carbohydrates, waxes, and resins include: an overview of what can be accomplished with the protocol, a flow chart detailing all major and supporting operations, a list of equipment and supplies needed, sample requirements, detailed descriptions of preparation and analysis procedures, and a discussion of how to interpret results. Additional protocols cover written documentation of TLC plates, photo-documentation of plates using visible or ultraviolet light, application of sample material to the plates, evaluation of plates, and the preparatory steps of acid hydrolysis of carbohydrates and vapor phase hydrolysis of proteins.

The authors note that other protocols may also be useful, and that the ones given may need to be modified to take into account the development of new chromatographic materials and methodologies or limitation of available materials and equipment. Thus, readers are encouraged not to be rigid in the application of the protocols but to modify them as needed. This is a theme found throughout the book—that it is desirable for an analyst to understand the theory and application of TLC well enough to feel comfortable modifying the protocols presented here or elsewhere in the literature in order to meet specific individual needs.

The protocols are provided to supplement descriptive text covering the analysis of specific categories of materials. Descriptions include discussions of relevant example analyses. Bibliographic references are made to literature in conservation, forensic science, and chemistry. The detailed glossary at the back of the book will be of special help to the beginning analyst.

This publication is certain to be useful in courses at conservation training programs where TLC is taught. It can also be used by conservators, conservation scientists, or other professionals conducting research on works of art, archaeological objects, or ethnographic artifacts. It will be helpful to those who wish to independently learn TLC or to supplement courses they have taken in chromatographic techniques with information directly pertinent to their work.

*Chandra L. Reedy*
*Professor, Ph.D. Program in Art Conservation Research*
*University of Delaware*

Part 1

# Handbook

# Chapter 1

## An Overview of Thin-Layer Chromatography

The purpose of this chapter is to present an overview of thin-layer chromatography (TLC), including:

- The relationship of TLC to other chromatographic techniques
- The history of TLC
- Important aspects of the method

Thin-layer chromatography is a separation technique that involves several steps. First, a solution made from a sample is applied to a coated plate. The carrier solvent of the sample solution evaporates and deposits the sample in a small spot or zone at the origin of the plate. The plate is then placed in a sealed vessel containing a small volume of an appropriate solvent mixture. As the solvent mixture travels up the plate by capillary action, the components from the sample travel up at different rates due to their interaction with the coating on the plate (the stationary phase) and the moving solvent system (the mobile phase). This process is called the development of the plate. The plate is developed to achieve separated spots or bands. The plate is then removed from the solvent system, and the components of the sample are visualized. This usually involves reacting the component with a reagent that produces visible or fluorescent spots when observed under either normal or ultraviolet light. This pattern of spots seen for the binder sample is called the chromatogram.

### Comparison of TLC to Other Chromatographic Methods

TLC is but one of a group of techniques that are based on chromatographic principles. Chromatography literally means "color writing." The term represents the early approach in chromatography by botanist Michael S. Tswett in 1903. Tswett separated red and yellow plant pigments from an original green extract of spinach leaves on a calcium carbonate column by adding an eluting solvent of petroleum ether and alcohol. Distinct colored bands or zones were seen on the column as the solvent flowed through it. In time, a number of different types of chromatographic techniques arose from a common principle (the distribution

of an analyte between a stationary and a mobile phase). Chromatography is essentially a physical method of separation in which components of a material are distributed between two phases. One phase is stationary (stationary phase) while the other (the mobile phase) percolates through it in a definite direction (Poole and Poole 1991). As the mobile phase moves, the components are displaced from the origin and separate from each other. A distinction between chromatographic techniques (see Figure 1.1) can be made based on the nature of the mobile and stationary phases (Grinberg 1990).

In general, the mobile phase can be a gas, as it is in gas chromatography (GC), or a liquid, as it is in high-performance liquid chromatography (HPLC) or planar chromatography (including TLC). The stationary phase is usually a porous solid of high surface area. It can be packed densely into a column or can be spread evenly on a planar support. The stationary phase can be chemically modified to change its reactivity or used as a support for a thin film of liquid. For TLC, the stationary phase is spread as a thin, homogeneous layer on a flat plate of glass or similar inert backing, and the mobile phase moves through the layer by the action of capillary forces.

TLC is generally regarded as a simple, rapid, and inexpensive method for the separation, tentative identification, and visual semiquantitative assessment of a wide variety of substances. In recent years, TLC has come to rival HPLC and GC in its ability to resolve complex mixtures and to provide quantitative results. The evolution of the technique has included improvements in the quality of the TLC plates and detection reagent application techniques, the introduction of new stationary phases and approaches in plate development, and the design of sample application equipment and densitometric scanning.

TLC is compared to other chromatographic techniques in Table 1.1 and is discussed here; additional details can be found in other sources (Fried and Sherma 1986a).

TLC offers many advantages over paper chromatography, which is limited to the use of cellulose as a stationary phase. TLC utilizes a range of sorbent layers that offer superior resolution, speed, and sensitivity.

**Figure 1.1.**

A general classification scheme of chromatographic techniques. GSC = gas-solid chromatography; GLC = gas-liquid chromatography; LSC = liquid-solid chromatography; LLC = liquid-liquid chromatography; BPC = bonded-phase chromatography; IEC = ion-exchange chromatography; EC = exclusion chromatography; TLC = thin-layer chromatography; PC = paper chromatography; GPC = gel-permeation chromatography; and GFC = gel-filtration chromatography.
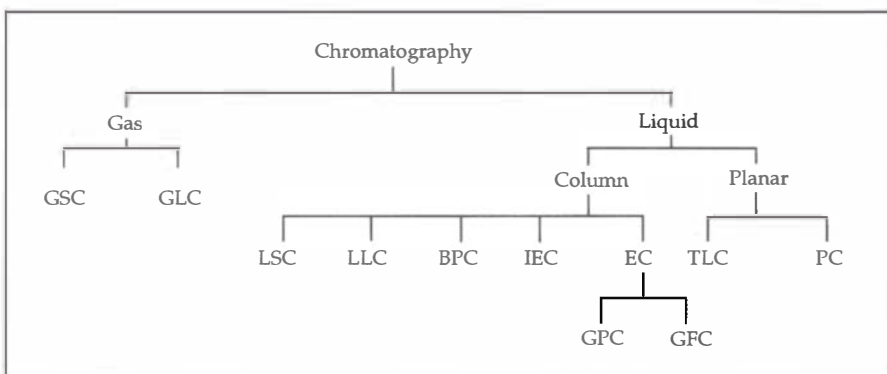
| | Thin-layer chromatography (TLC) | Paper chromatography (PC) | High-performance liquid chromatography (HPLC) |
|---|---|---|---|
| Methodology | Planar technique using thin-layer sorbent; many types of sorbents are commercially available | Planar technique limited to paper as a stationary phase | Closed column system; samples are introduced sequentially into a mobile liquid; the stationary phases available are similar to those used in TLC |
| Sample Size | 500 µg to 1 mg | 500 µg to 1 mg | 1 µg to 10 µg |
| Cost | Minimal equipment and chemicals needed, which leads to low cost | Low cost | Requires specialized instrumentation for analysis and needs larger volume of solvents, which lead to much higher cost |
| Speed | Up to 72 samples can be separated on one plate using a horizontal chamber, which leads to higher sample throughput and lower analysis time | Up to 18 samples on a 20 x 20 cm sheet of TLC paper | Number of samples limited to elution time of each sample, since the samples are introduced into the system one at a time |
| Resolution | Superior to PC and lower or equal to HPLC, depending on system used | Lower resolution | Superior to TLC and PC |
| Sensitivity | Lower sensitivity than HPLC | Lower sensitivity than HPLC, and in some cases lower sensitivity than TLC | Much higher sensitivity due to the use of instrumental detection, such as flame ionization detection (FID) |

Table 1.1.

A comparison of thin-layer chromatography, paper chromatography, and high-performance liquid chromatography.

HPLC and TLC are similar in that the mobile phase, the stationary phase, and the separation mechanism are identical. While HPLC and TLC are considered complementary techniques, HPLC is considered more efficient than TLC in separating components. Also, an HPLC system is a closed system that allows for greater control of the mobile phase velocity. Advantages of TLC over HPLC include higher sample throughput due to simultaneous analysis of samples, and the flexibility and versatility of development and detection steps. More solvents can be used as mobile phases in TLC because the solvent is completely evaporated before detection and the plate is used only once. Also, TLC uses a much smaller amount of solvent for each analysis, minimizing the costs of solvents and waste disposal.

The main advantages of TLC are its low cost and the relative speed of analysis. The materials needed to perform TLC are minimal. They include a development chamber, chromatographic plates, solvents, detection reagents, and reference materials. Also, TLC can be applied to the detection and identification of a wide range of materials, like those found in binding media.

Disadvantages of TLC analysis include the need for a larger sample size and its lower sensitivity in comparison with other methods, such as HPLC or GC. For binding media analysis by TLC, the sample size is usually 500 μg. For paint samples that contain a low binder concentration, samples up to 1 mg in size may be needed.

## The History of TLC

The development of modern thin-layer chromatography has its beginnings in liquid chromatography. Like most scientific methods, chromatography evolved from initial phenomenological observations, through early empirical research and the study of the underlying theoretical principles, and finally, advancements in technique. While the Russian botanist Tswett is generally credited with the discovery of chromatography for his work in the separation of plant extracts on a column of sorbent, others observed the phenomenon of separation before Tswett. For example, in 1844 C. Matteucci observed the rings left by a drop of chocolate on a piece of paper. In 1850 the German dye chemist F. F. Runge recognized the possibility of separating inorganic ions when he observed their migration through paper. He initially described the forces responsible for the separation as the "living forces," but later attributed the phenomenon to capillary forces (D'Ascenzo and Nicolini 1990). Tswett's contribution to chromatography was the understanding of adsorption, which led to chemical separation by liquid column chromatography.

Although chromatographic techniques were little used for the next thirty years, in the 1930s they were reintroduced by biochemists. In 1941, A. J. P. Martin and R. L. M. Synge introduced partition chromatography. They found that they could separate amino acids successfully if a water phase was held stationary by adsorbing it on silica gel, while permitting a mobile chloroform phase to flow over it. Thus they described the use of a liquid stationary phase with a liquid mobile phase and suggested that a gas might also be used as the mobile phase. In 1952, Martin and Synge were awarded a Nobel Prize in chemistry for their theoretical development of partition chromatography (Brooks 1987).

The development of TLC can be divided into three eras: the early years (1938–1951), the classical period (1956–1980), and the modern period (1981 to the present). Figure 1.2 shows a time line for the history of TLC.

**The Early Years: 1938–1951**

1844 — C. Matteucci observes the separation of chocolate on paper.

1850 — F. F. Runge separates inorganic ions on paper.

1903 — M. S. Tswett, a Russian botanist, separates colored plant pigments on a column of calcium carbonate.

1938 — N. A. Izmailov and M. S. Shraiber use thin layer on microscope slides.

1941 — A. J. P. Martin and R. L. M. Synge introduce partition chromatography, for which they receive a Nobel Prize in chemistry in 1952.

M. O'L. Crowe uses thin layer in a petri dish for separation of plant extracts.

1947 — J. W. Sease adds fluorescent detector to sorbent layer for detection of colorless compounds.

1949 — J. E. Meinhard and N. F. Hall improve thin-layer chromatography by adding binders to adhere alumina to microscope slides.

J. G. Kirchner and colleagues introduce and improve sorbent layers for TLC.

1953 — J. M. Miller and J. G. Kirchner develop numerous visualization reagents for in situ detection of separation zones.

**The Classical Period: 1956–1980**

1956 — Egon Stahl introduces silica gel as a sorbent layer and publishes his work titled *Thin-Layer Chromatography*.

1958 — Stahl works with manufacturers to introduce commercial materials for TLC.

1962 — *Thin-Layer Chromatography: A Laboratory Handbook* is edited by Stahl, who popularizes the technique.

A variety of development chambers is introduced, including linear tanks and horizontal chambers.

Instruments for scanning densitometry appear.

1975 — Commercial production of high-performance TLC plates leads to improvements in practice and instrumentation.

1979 — New methods of sample application are introduced, including the contact spotters by D. Fenimore.

New approaches to plate development appear. Pressurized ultramicro chamber is introduced.

**The Modern Period: 1981–present**

1984 — Automated methods of plate development are created, including the Automated Multiple Development System (K. Burger).

Sophisticated methods of detection are introduced by coupling TLC with other analytical methods.

1985 — Methodology for coupling TLC to Mass Spectroscopy is first described.

1990 — Flame ionization detection is used for samples of low volatility that lack a chromaphore for detection. Separation is performed on quartz rods coated with a sorbent sintered into the surface of the rod.
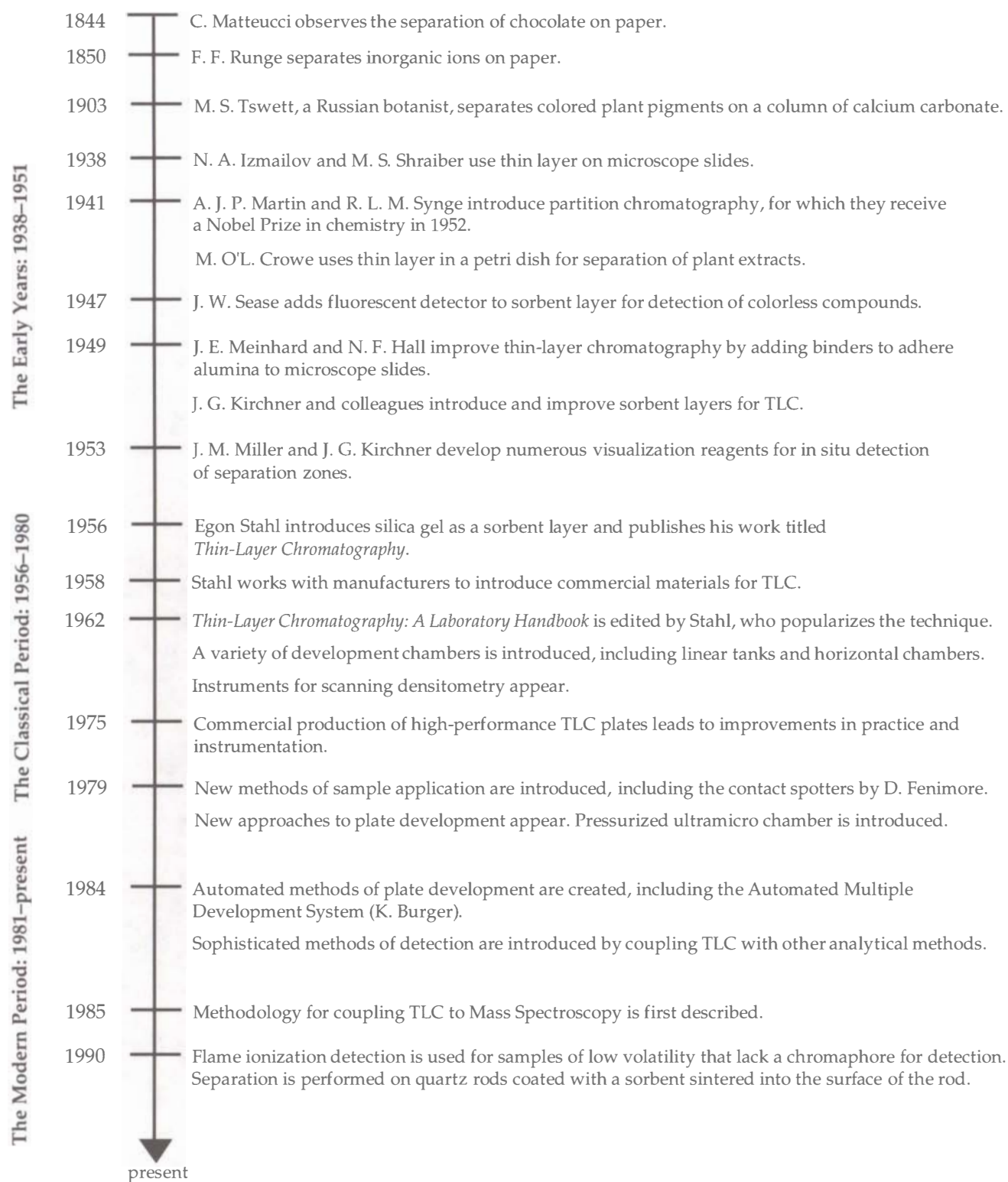
present

**Figure 1.2.**

A time line for the history of thin-layer chromatography.

**The Early Years: 1938–1951**

The first reported use of a thin layer was in 1938 by two Russian workers, N. A. Izmailov and M. S. Shraiber. They separated plant extracts on a slurried adsorption medium spread to a 2-mm-thick layer by spotting an alcoholic plant extract in the center of the layer and observing rings as the solution spread. Adsorption media that they tried included chalk, talc, magnesium oxide, lime, and aluminum oxide. This method is now called circular chromatography. They found that the results obtained by this method were qualitatively the same as those obtained by the usual chromatographic adsorption method of analysis. This work was later reviewed in 1941 by M. O'L. Crowe, who reported that he and his colleagues had been using a thin layer of adsorbent in a petri dish and achieving similar results (Stahl 1969a).

The fledgling technique was then improved by the addition of binders to the sorbents. This was first reported in *Analytical Chemistry* by J. E. Meinhard and N. F. Hall in 1949. They used a binder to adhere alumina to microscope slides, and separated inorganic ions by drop chromatography. At about the same time, J. G. Kirchner and his colleagues at the U.S. Department of Agriculture were working to determine the chemistry of orange and grapefruit flavors. They initially attempted to separate the chemicals that produced flavor by using paper chromatography. They also experimented with impregnating the paper with silicic acid. Kirchner had a habit of clipping interesting abstracts out of *Chemical Abstracts*. One day, when one of Kirchner's colleagues was quite frustrated about a difficult separation, Kirchner reached across his desk, picked up the abstract of Meinhard and Hall's work, and said, "Try this" (Kirchner 1979). After experimenting with the findings in the abstract, Kirchner and his colleagues found that silicic acid bound with amioca starch gave a satisfactory layer for TLC. He continued his work with sorbent layers on glass plates and developed TLC essentially as we know it today (Fried and Sherma 1986b). Kirchner also observed that in order to obtain reproducible results, conditions had to be standardized. For instance, he showed that silicic acid must be screened to remove coarse particles, and that it is necessary to run standards to verify that the layers are properly prepared. Standards also serve as references when comparing $R_f$ values (see page 14).

Another early improvement to the technique was the methodology developed to assess the chromatogram. Colored separated zones are visible, but many substances are colorless. To cope with this problem, research on the detection of chromatographic zones progressed in two main directions: physical methods and chemical methods. The addition of an ultraviolet fluorescing agent to the thin layer, an example of a physical method, was first reported by J. W. Sease (Sease 1947). Numerous spray reagents were then studied for other compounds. These spray reagents aided in the detection of chromatographic zones by chemical reaction with the substances. In situ reactions, including oxidations, reductions, hydrolysis reactions, and the preparation of derivatives, were reported by J. M. Miller and J. G. Kirchner in 1953.

**The Classical Period: 1956–1980**

Perhaps more than any other scientist, Egon Stahl advanced the technique of thin-layer chromatography. Stahl was born in 1924 in Germany. He was the author of more than 150 scientific papers on the components of medicinal plants, chromatography, and related scientific methods. After World War II, Stahl analyzed drugs, tinctures, and medicinal plant extracts for a pharmaceutical company. This work led him to the use of paper chromatography. Later, he realized that layers with very small pores and fine grains were needed to separate some chemical compounds. First, he tried using cigarette paper, and later experimented with magnesium grooves and rods and aluminum oxides. He succeeded in his separations when he spread fine-grained aluminum oxides and silica gel onto glass plates. While he knew that this new form of chromatography was very similar to column chromatography, he slowly came to understand additional influences, such as the thickness of the layer, the length of the run, chamber saturation, and other conditions. In 1956, he published a first work with the title "Thin-Layer Chromatography" in a professional magazine called *Die Pharmazie*. Thus, the technique was named, but it did not immediately find wide acceptance.

Stahl lists the following reasons for this lack of acceptance:

> I asked myself why it did not find acceptance and thought the following reasons responsible: absence of commercially available standard adsorbents of narrow range of grain size for thin-layer chromatography; absence of suitable equipment for preparing thin layers; and absence of suitable examples stimulating the use of the method (Stahl 1979).

He kept these points in mind as he continued his research. In 1958, a basic kit for TLC was manufactured by DESAGA, and "silica gel plates according to Stahl for TLC" were manufactured by E. Merck. At this point, the method became more popular, and by 1962 so many papers had been published that Stahl edited the first laboratory handbook on TLC, *Dunnschlicht-Chromatographie, ein Laboratoriumshandbuch*. A second edition, which was translated into English in 1967, contained more than one thousand pages (Stahl 1969b). A major breakthrough in the field came in the early 1960s when convenient precoated plates became commercially available.

This was also a period of time when a large variety of TLC chambers were designed for various applications. These chambers included tanks for ascending, descending, and horizontal development, and tanks for electrophoresis. Linear development tanks for ascending development included the twin-trough N chamber, which has a glass ridge down the center, and the sandwich chamber, also called the S-chamber, which has a second glass plate about 1 mm from the surface of the TLC plate. Some commercially available sandwich chambers can be used in the horizontal mode of circular development. Another type of horizontal chamber, the BN chamber used for the continuous flow technique, was described by M. Brenner and A. Niederwieser in 1961.

Instruments for scanning densitometry using absorbance or fluorescence measurements in the reflectance or transmission mode first appeared in the mid-1960s. This was initially reported by M. S. J. Dallas et al. (Dallas et al. 1964) and K. Genest (Genest 1965). Since that time, densitometry has undergone continuous changes, especially with the introduction of new technology such as solid-state video cameras and image processing (Belchamber and Brinkworth 1988).

In the mid-1970s, the commercial production of high-performance TLC (HPTLC) plates provided the impetus for improvements in practice and instrumentation. These high-performance TLC plates differ from conventional TLC plates in a number of ways, but mainly in the size of the particles used in the manufacture of the plates and the need for a much more precise and instrumentalized approach in order to obtain the best results (Dallas et al. 1988). This development led to methods termed "high-performance TLC," by A. Zlatkis and R. E. Kaiser (1977).

## The Modern Period: 1981–Present

After a decline in the reported use of TLC in the 1970s, there was renewed interest in the technique. (The decline corresponded to the period of development of the HPTLC method.) The "renaissance" of the TLC technique may have been related to improved methodology, with an emphasis on the instrumentation and automation of operations. Other developments have included new approaches in TLC plate development and the coupling of TLC with spectrometric methods.

Automation of operations in TLC has focused on the particular steps with the greatest potential for error. These included application of sample solutions, chromatographic development, detection, and quantitative in situ evaluation. In order to use the power of HPTLC plates, new methods of sample application were introduced, including the contact spotter by D. Fenimore (Fenimore and Meyer 1979) and the Automatic TLC sampler (CAMAG, Switzerland). One automated method derived by K. Burger (1984), the Automated Multiple Development system (AMD), resulted in a dramatic improvement of chromatographic selectivity. This apparatus can be used for one to twenty-five individual developments. The procedure involves very small developing distances, with each development being longer than the previous one by 3–5 mm. Between each development, the solvent system is completely removed and the plate is dried under vacuum. The solvent system can be changed with each development. Improvements in the instrumentation of scanning densitometers were also seen during this era.

New approaches in the development of the TLC plate have focused on the transport of the mobile phase through the stationary phase. Conventional TLC relies on the rise of the mobile phase by capillary action, which can take up to several hours depending on the TLC system. AMD is also based on capillary action for the transport of the mobile phase. Several new techniques based on an alternative approach have been developed. Forced flow techniques can be subdivided into those where the mobile phase is controlled in an open system, and those where the flow is pressurized in a closed system. Centrifugal

systems are an example of a controlled mobile phase in an open system and have been used in the development of TLC plates. The sample to be separated is spotted in the middle of the plate. The solvent system is supplied at the center of the spot, and the mobile phase then moves outward by centrifugal forces as the plate is spun. Pressure can also be used to alter the transport of the mobile phase, similar to HPLC methods (Ferenczi-Fodor et al. 1991). The first step in the development of a planar version of HPLC came with the development of a pressurized ultramicro chamber, the basic instrument for overpressured layer chromatography (OPLC) (Tyihak et al. 1979). In OPLC, a pump system is used for the admission of the solvent system.

Sophisticated methods of detection have been introduced by coupling TLC with spectroscopic methods, such as infrared spectroscopy (TLC/FTIR) or mass spectroscopy (TLC/MS) (Jork 1993). As early as 1964, it became feasible to record the reflectance spectra of the separation zones in the ultraviolet and visible ranges of the spectra. This methodology was extended to the infrared spectra by coupling TLC to Fourier transform infrared spectroscopy. A diffuse reflectance infrared spectroscopy unit has been specifically designed for this purpose (Bruker Instruments, Karlsruhe, Germany), and targeted quantitative determinations can be made at a wavelength that is specific for a particular compound. Methodology for the coupling of TLC to mass spectroscopy was first described in 1985 (Chang and Andrawes 1985). In this technique the zone is removed from the TLC plate and transferred to the mass spectrometer. Continued research in this area brought about interface systems for on-line coupling of TLC to mass spectroscopy.

## Theoretical Aspects of Thin-Layer Chromatography

Descriptions of the general theory of thin-layer chromatography can be found in the literature (Kowalska 1991; Poole and Poole 1991; Fried and Sherma 1986a; Brenner et al. 1965). Presented here is a summary of important theoretical aspects that are valuable to an understanding of chromatographic separation by TLC.

The main purpose of TLC is to separate or resolve components of mixtures. As applied to the analysis of binding media, the sample is a mixture of chemical building blocks or compounds. The chromatogram of the sample can be characterized by the number and location of spots or zones. By comparing sample chromatograms to chromatograms of known reference materials, the sample may be identified.

Chemical separations by TLC result from the interaction of molecules with the stationary and mobile phases. The developing solvent system or mobile phase is the transport medium for components of samples to be separated as they migrate through the stationary phase by capillary forces. As the solvent system moves upward through the plate, the components are affected by two opposing forces, the driving force of the mobile phase and the resistive or retarding action of the sorbent. The driving force tends to cause the components to move

in the direction of the flow of the mobile phase, and the resistive forces impede this movement by pulling components out of the flow of the mobile phase and holding them on the stationary phase by adsorption. Thus, each molecule follows a "stop-and-go" path through the sorbent layer. At the end of development, each component spot has traveled a certain distance. Components that are more strongly attracted to the sorbent layer will travel a shorter distance, while components that are more soluble in the mobile phase will travel a longer distance from the origin. The spots become larger in size due to fluctuations in the movement of the individual molecules (Fried and Sherma 1986c).

The migration of the components in a chromatogram can be characterized by a basic parameter called the $R_f$ **value**. It is calculated as the ratio of the distance moved by the solute (component), to the distance moved by the mobile phase front (Sherma 1991). This can be expressed as:

$$Rf = \frac{\text{distance moved by the solute}}{\text{distance moved by the mobile phase front}}$$

The distance moved by the solute is measured from the origin to the center of the zone. An illustration of the calculation of an $R_f$ value is shown in Figure 1.3. Identification of compounds by TLC is based on the comparison of chromatograms of known and unknown materials. $R_f$ values facilitate this comparison and are used as guides to the relative migration and sequencing of components within a mixture. It should be noted that there are many factors that can cause variance in the $R_f$ values of a chromatogram, therefore known materials must be run next to unknown samples on the same chromatographic plate for comparison.

A measure of the selectivity of a TLC system is the **resolution** of two chromatographic zones. The resolution $(R)$ is defined as the distance between two zone centers $(d)$ divided by the average widths $(W)$ of the zones:

$$R = \frac{d}{(W_1 + W_2)/2}$$



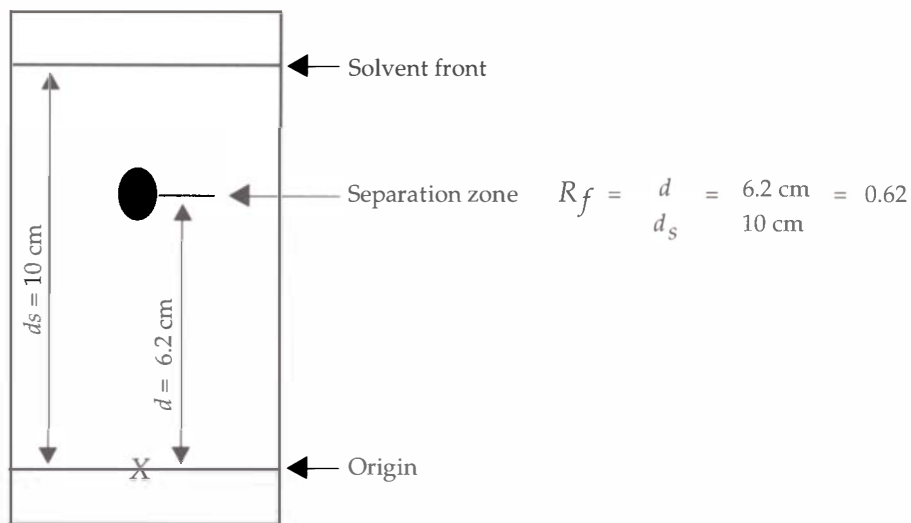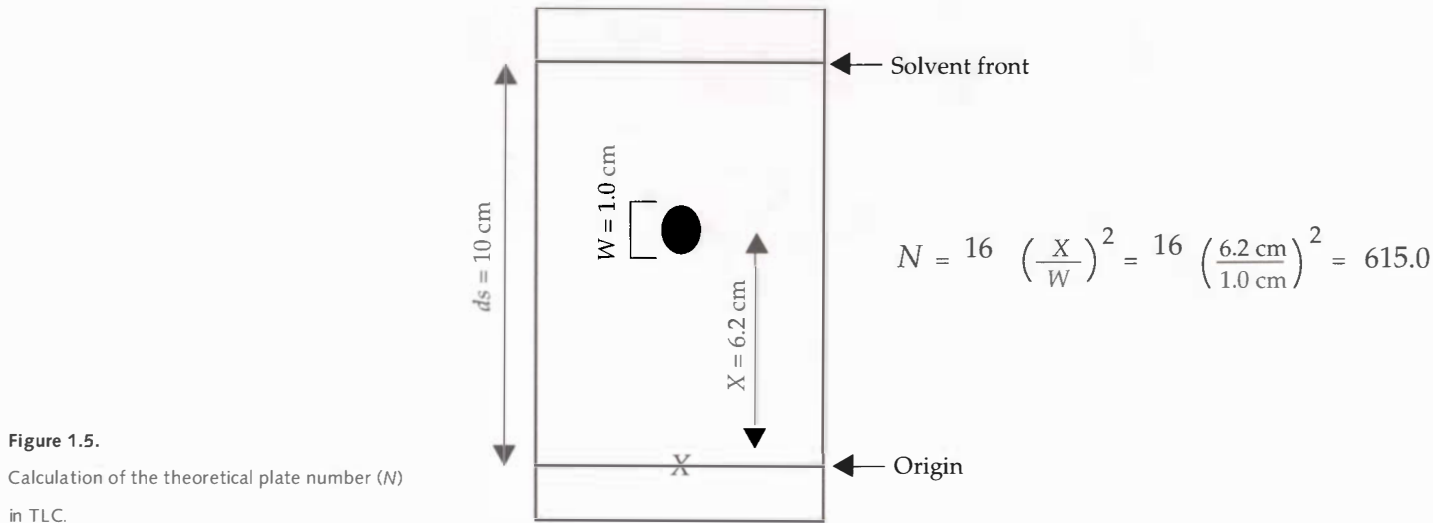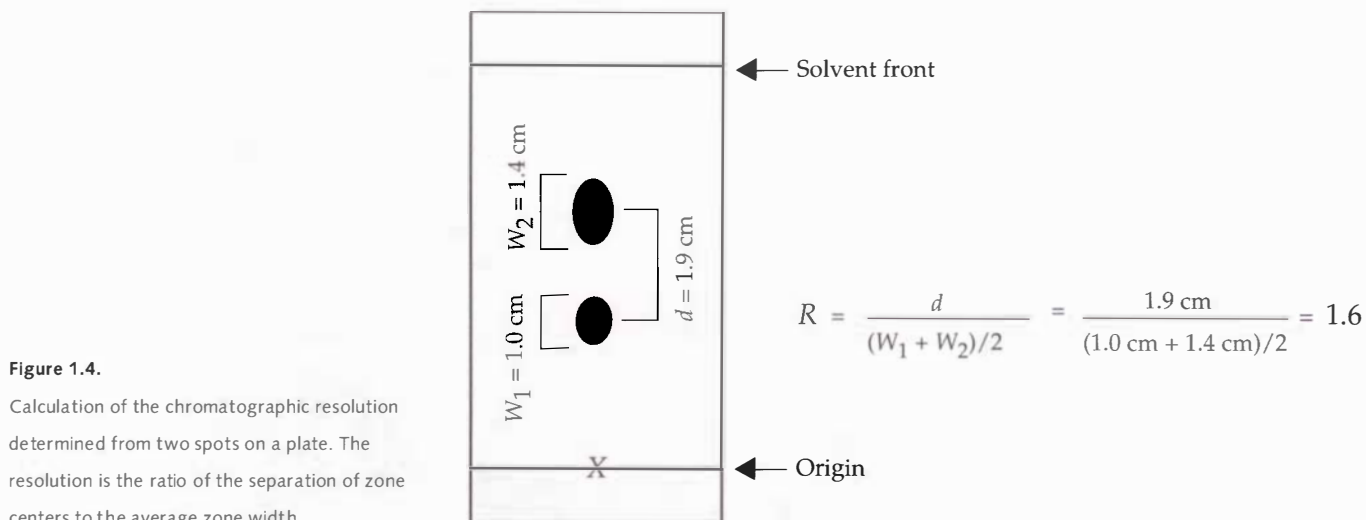$$R_f = \frac{d}{d_s} = \frac{6.2 \text{ cm}}{10 \text{ cm}} = 0.62$$

**Figure 1.3.**

Calculation of the $R_f$ value in TLC.

An example of this calculation can be seen in Figure 1.4. The resolution of the system can be improved by moving the zones farther apart (increasing the selectivity). The resolution can also be improved by decreasing the widths of the zones by improving the efficiency of the system.

The efficiency of a TLC system is measured by a parameter called the **theoretical plate number** ($N$). This value is useful for comparing chromatographic methods as well as the efficiency of individual TLC systems. It is calculated from the equation:

$$N = 16 \left( \frac{X}{W} \right)^2,$$

where $X$ is the distance (in mm) from the origin to the center of a given zone and $W$ is the width of the zone. An example of this calculation is shown in Figure 1.5. A large value of $N$ indicates a high-efficiency TLC system with tight zones.



$$R = \frac{d}{(W_1 + W_2)/2} = \frac{1.9 \text{ cm}}{(1.0 \text{ cm} + 1.4 \text{ cm})/2} = 1.6$$

**Figure 1.4.**

Calculation of the chromatographic resolution determined from two spots on a plate. The resolution is the ratio of the separation of zone centers to the average zone width.



$$N = 16 \left( \frac{X}{W} \right)^2 = 16 \left( \frac{6.2 \text{ cm}}{1.0 \text{ cm}} \right)^2 = 615.0$$

**Figure 1.5.**

Calculation of the theoretical plate number ($N$) in TLC.

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Chapter 2

**Technique of Thin-Layer Chromatography**

The purpose of this chapter is to present:

- An introduction to the technique of thin-layer chromatography
- A brief description of the steps involved in performing a chromatographic separation

The process of thin-layer chromatography involves the series of steps shown in Figure 2.1. These steps include sample preparation, selection of the chromatographic plate, selection of the mobile phase, application of the sample to the plate, development of the plate, drying of the plate, detection of the separation zones, visual examination, and documentation.

**Sample Preparation**

The first step is the preparation of the sample, which varies depending on the type of material being analyzed. For binding media analysis, two principal methods of sample preparation are used: dissolution of the sample in an appropriate solvent (for waxes or resins) or acid hydrolysis of the sample into its components (for proteins and carbohydrates). The choice of the solvent is dependent on the solubility of the sample. The properties of the carrier solvent are also important since it is the vehicle used to apply the sample to the plate.

For waxes and resins, the sample is dissolved in a carrier solvent, then applied to the plate. A good solvent for the dissolution of the sample is one that readily dissolves all of the components of interest. Tables of solubility are listed for film substances in various organic solvents (Gettens and Stout 1966). From this information, chloroform was chosen for the dissolution of waxes, and ethyl acetate was chosen for the dissolution of resins.

For proteins or carbohydrates, the sample must be broken into its components through the use of acid. A summary of acid hydrolysis is given here, and more detailed descriptions are given in Chapters 4 and 5. The apparatus for acid hydrolysis is shown in Figure 2.2. This equipment includes a preheated oven, gas manifold, vacuum pump, and special reaction chambers (Pierce vials and Miniert valve caps). Small
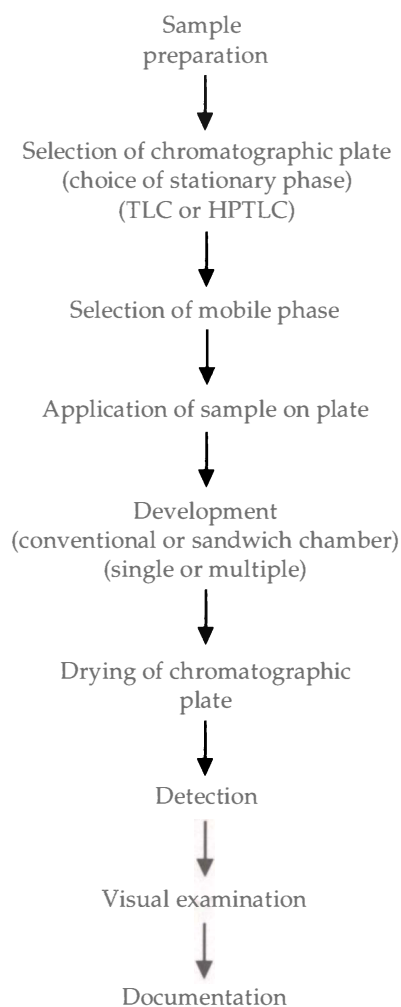
Sample
preparation

↓

Selection of chromatographic plate
(choice of stationary phase)
(TLC or HPTLC)

↓

Selection of mobile phase

↓

Application of sample on plate

↓

Development
(conventional or sandwich chamber)
(single or multiple)

↓

Drying of chromatographic
plate

↓

Detection

↓

Visual examination

↓

Documentation

**Figure 2.1.**

The process of thin-layer chromatography.

glass vials with preweighed binding media samples are placed in the Pierce reaction chamber. After acid is added to the sample, a vacuum is pulled through the Pierce Miniert valve to remove air that might cause oxidation when the sample is heated. The reaction chamber is placed in a preheated, 105 °C oven, and the samples are heated 5–24 hours, depending on the concentration of the acid being used in this step. After acid hydrolysis, each sample is dried under a stream of nitrogen, again to prevent oxidation, which flows through a needle from the gas manifold into the tube and over the sample. Once dry, the samples are reconstituted in a carrier solvent such as methanol.

The acid hydrolysis may be performed as described above (carbohydrates) or through vapor phase hydrolyzation (proteins), where acid is added to the bottom of the reaction chamber, but not to the sample vials.

## Selection of the Chromatographic Plate

The second step in the process of TLC is the selection of the chromatographic plate (see Chapter 3, pages 26–28, for discussion of sorbent layers).
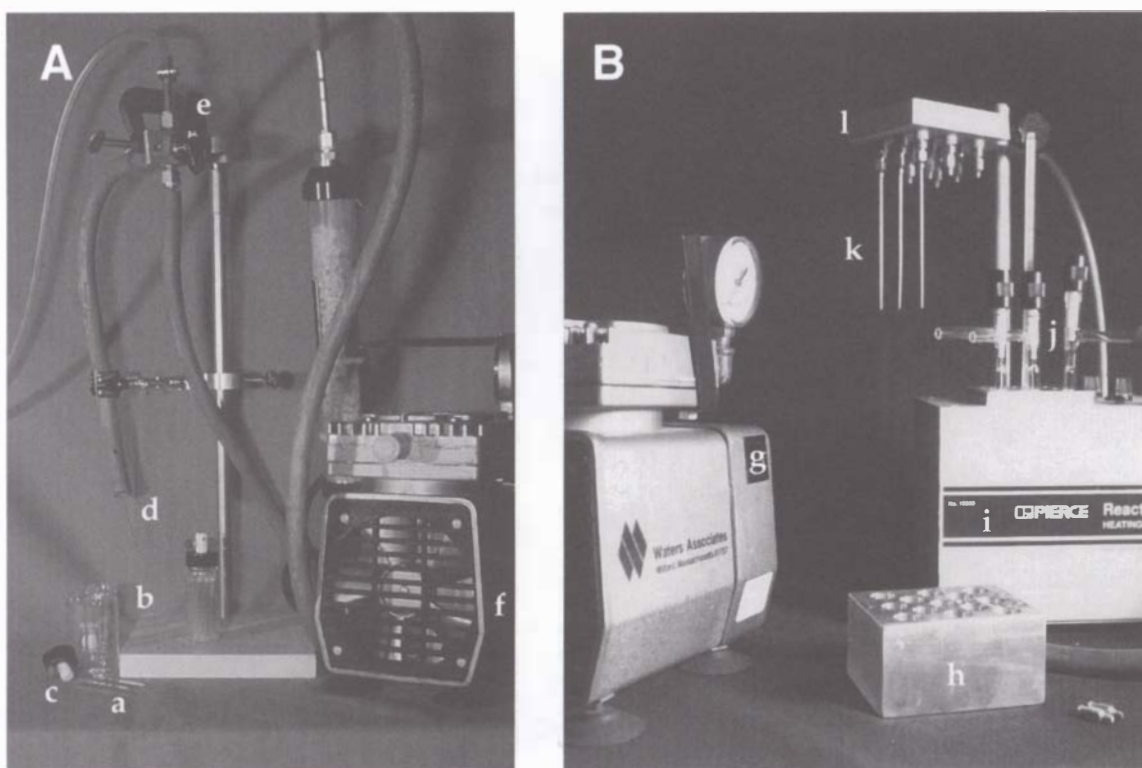
**Figure 2.2.**

Two types of hydrolysis equipment. On the left is a system composed of various laboratory supplies that can be used to perform vapor phase or conventional acid hydrolysis. A sample is weighed directly into 1-ml vials (a). The vials are placed into a reaction chamber (b). Acid is added to the chamber or the samples; then the chamber is sealed with a cap containing a Miniert valve (c). The reaction chamber is flushed and placed under vacuum for three cycles. This can be done by inserting a needle (d) that is connected via tubing to a two-way valve (e). The valve can be directed to a vacuum pump (f) or a tank of nitrogen (not shown). On the right is a commercially available system manufactured by Pierce Corp. The Pierce Reacti-Therm sample incubation unit is used for conventional acid hydrolysis of proteins and carbohydrates. Components of this system include: (g) vacuum pump, (h) aluminum heating block, (i) heating unit, (j) vacuum hydrolysis tubes, (k) needles, and (l) gas manifold.

Resolution of the components of a sample depends on both the stationary phase and the mobile phase chosen for the analysis. Silica gel, an excellent stationary phase for many applications, is widely used in thin-layer chromatography. Prepared plates of silica gel are commercially available from several manufacturers. It is important to note that variances in the manufacture of a TLC plate can greatly affect the separation of the components. To avoid problems of this sort, the plates are purchased from the same manufacturer and prepared consistently for better reproducibility. The plate is usually prewashed in methanol and activated by heating.

There are two basic types of thin-layer chromatographic plates, conventional (TLC) and high-performance (HPTLC). The major differences between TLC and HPTLC plates are the particle size, the size distribution of the particles, and the thickness of the stationary phase. HPTLC plates have optimum particle sizes and thinner layers that result in a higher efficiency in the separation of components. Analyses using HPTLC plates require less development distance, and therefore less time.

One drawback can be the low volume of sample that must be loaded onto the HPTLC plate for optimum performance, as large amounts of sample on the plate result in a poorer separation.

### Selection of a Solvent System

A key step in designing a TLC application is the choice of the solvent system, or mobile phase (see also Chapter 3, pages 28–31). While there are several theories on the optimization of solvent systems (DeSpiegeleer 1991), the selection of solvents for this work was based on testing systems noted in the literature (Bruno et al. 1989; Stahl 1969).

### Spotting the Sample

The fourth step in the process is the spotting of the sample solution to the prepared TLC plate. The sample solution is drawn into a glass capillary tube micropipette. A volume of 0.1 to 1.0 µl of the sample solution is spotted on the baseline of the plate. A template or ruler is often used as a guide so that the spots are equally spaced along the baseline. The application of the spot is an important step often requiring practice to obtain optimally small, round sample spots. One method for obtaining small spots on the origin involves applying portions of the total volume and drying the spot with an air gun (cool setting) between each application.

### Development of the TLC Plate

After the sample spots dry, the plate is placed in a development chamber that contains a solvent or mixture of solvents (for a detailed description of different types of chambers, see Chapter 3, pages 31–34). There are two types of chambers used in the analysis of binding media: the conventional chamber and the sandwich chamber. The conventional chamber is easiest to use, although it must be presaturated with solvent vapors. The process usually takes 15–30 minutes. This type of chamber requires approximately 30 ml of solvent system.

     The sandwich chamber is often used with HPTLC plates and uses a much smaller volume of the solvent system. The chromatographic plate is attached to the backing plate so that there is a minimal vapor space between the plates. It is then placed in the trough of the sandwich chamber, which holds about 5 ml of the solvent system. Since the vapor space is minimized, less solvent evaporates from the surface of the chromatographic plate; thus, less solvent can be used. One disadvantage of the sandwich system is that highly volatile solvent systems may evaporate from the chamber before the development is complete. Also, the solvent front may become uneven as a result of preferential evaporation at the edges of the plate caused by air currents. To decrease

the evaporation of the volatile solvents and to keep the vapor space saturated, the entire sandwich chamber is placed inside a tightly sealed vessel.

The plate is developed for a specified distance, usually 8 cm for the 10-cm HPTLC plates in a sandwich chamber, and 17 cm for the 20-cm TLC plates in a conventional chamber. Then the plates are removed from the chamber and allowed to dry at room temperature in the fume hood. This drying process is dependent on the solvent system used, but usually takes about 30 minutes.

## Detection of Separation Zones

The chemical components of the sample are separated on the TLC plate. They may be colored or colorless compounds. If they are colorless they must be made visible in some manner. The detection of the individual components is aided by reacting the components with a chemical reagent to form visible spots under normal or ultraviolet light (additional information on physical and chemical methods of detection is presented in Chapter 8, pages 67–73). The detection reagent can be applied by dipping or spraying. Spraying is the most commonly used method, since it requires minimal amounts of the reagent. Reusable spray bottles dispense an ultrafine mist that is ideal for the visualization of components on a TLC plate.

## Visual Examination

After detection, the plate is examined to determine the location of the components. The plate is first viewed under normal light. Any discoloration that may indicate separation zones is marked lightly with pencil. Next, the plate is examined under ultraviolet light. Two types of UV light are used, a range of short-wavelength UV light (centered at 254 nm) and a range of long-wavelength UV light (centered at 366 nm). Any fluorescence is again marked with a pencil.

## Documentation

The chromatogram is carefully documented. The documentation includes a record of the materials analyzed, the type of plate used, the solvent system used, the type of chamber, and other conditions. In addition, a photocopy (for the lab notebook) and photographs (for permanent documentation) of the TLC plate are made. The plate is photographed on both black-and-white print and color slide film. Figure 8.6 (page 75) shows a simple chamber used to photograph TLC plates under ultraviolet light. It is constructed of a cardboard box, with panels inserted to support UV lamps at 45° angles to the plate (a description of techniques for documentation is given in Chapter 8, pages 73–75).

Finally, the location of each spot is measured and recorded, and the respective $R_f$ values for each component are calculated.

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# Chapter 3

## Methodology for Thin-Layer Chromatography

This chapter provides detailed information needed to develop TLC systems for use on samples, in general, and binding media, adhesives, and coatings, in particular. Sections within this chapter present:

- A report on the types of sorbent layers available and their interactions with both the solute and the solvent system
- A discussion of solvent systems
- A detailed description of development chambers and techniques of development
- An introduction to detection methods
- A prelude to documentation needed for TLC analyses

Conventional thin-layer chromatography requires minimal equipment and supplies, including pipettes for sample application, a plate coated with a thin layer of sorbent, the appropriate solvent system and a development chamber for the application, a means of detecting the resulting chromatogram, and equipment to document the chromatogram. The equipment can be simple, such as a screw-top jar for a developing chamber, or complex, such as a scanning densitometer for the quantitative analysis of a chromatogram. This chapter presents the basic equipment needed for the analysis of the organic binders, adhesives, and coatings found on artifacts. It also details some of the underlying theories for the choice of equipment and the development of new TLC systems. In addition, brief descriptions of more sophisticated methods will be given.

A TLC system consists of the sample or samples, the mobile phase, the stationary phase, the development chamber, and the detection reagent. The design of a thin-layer chromatographic system starts with an understanding of the chemical nature of the sample. Is the sample a mixture of large molecules, as in the case of proteins? Does the sample need to be broken into smaller units? Is the sample polar or nonpolar? From this starting point we begin to think about choosing appropriate stationary and mobile phases. These choices require an understanding of the interaction of the sample with the solvent system and stationary phase, as well as an understanding of the mechanisms involved in the separation by thin-layer chromatography. Once the solvent system and the chromatographic plate are selected, the development chamber is

chosen. After the plate is developed, the chromatogram is assessed. This requires visualization of the individual components of the sample that are separated on the plate, usually by chemically reacting the components with a detection reagent.

There are four mechanisms of separation that may occur during TLC: adsorption, liquid-liquid partition, ion exchange, or steric exclusion (Gocan 1990). The two main mechanisms—adsorption and partition—will be discussed here.

**Adsorption** is the process by which the solvent and the sample compete for reactive sites on the stationary phase (usually a polar sorbent). Components of the solute will compete more effectively or less effectively than the solvent, depending on the polarity of both the component and the solvent. For example, the most commonly used sorbent is silica gel. On the surface of a silica gel layer is a network of polar -OH groups bound to an $SiO_2$ skeleton. Adsorption on silica gel involves hydrogen bonding between functional groups on the sample and the -OH groups of the silica gel. Some molecules bond better to the -OH groups than others, and will travel a shorter distance from the origin. Those that bond more weakly to the -OH groups of the silica gel will travel a farther distance from the origin.

**Liquid-liquid partition** is the process that involves a liquid stationary phase bound to a solid sorbent. The components of the sample will spend a portion of the migration time in the liquid stationary phase and a portion of the time in the liquid mobile phase. Separation occurs when the components reside in both phases and have different retentions. The liquid-liquid partition mechanism can also take place on silica gel, under slightly different conditions, depending on how the plate is prepared (i.e., activated or not activated). Water binds to silica either as "capillary" water or as water associated with the -OH groups of the surface. This water can act as a stationary phase bonded to the sorbent.

When the stationary phase is polar and the mobile phase is less polar or nonpolar, the system is called **normal phase TLC**. If the sorbent is chemically altered (e.g., by bonding nonpolar groups to the surface of the silica gel or by coating or impregnating the stationary phase with a nonpolar organic solvent), a nonpolar stationary phase can result (Gasparic 1992). If this nonpolar stationary phase is used with a polar mobile phase, the system is called a **reversed phase (RP) system**. One of the advantages of chemically bonded phases is the almost unlimited variety of commercial or laboratory-prepared plates with a wide range of polarities.

## Sorbent Layers

A chromatographic plate is an even layer of a sorbent bound to an inert backing with a binder. The sorbent layer either plays an active role in the interaction with the solute (adsorption chromatography) or supports a liquid stationary phase (partition chromatography). At one time, chromatographic plates were made in the laboratory as needed, but the

quality of these plates could vary considerably. Quality TLC plates are now commercially available with a variety of sorbent layers. This section presents the components of the chromatographic plate, a comparison of TLC and HPTLC plates, and details the use of sorbent layers used in the analysis of organic materials from cultural objects.

The components of a TLC plate are the support, the sorbent binder, the sorbent layer, and sometimes chemical additives that aid in detection of the separation zones. The **support** of the thin layer is most commonly a glass plate. Other materials used as a support include thin plastic sheets and aluminum foil. The glass plate offers the advantage of being the most inert backing material; it is also a rigid planar support. The size of the plate is typically 20 × 20 cm. Other sizes include 5 × 20 cm, 10 × 20 cm, 10 × 10 cm, and "micro" (microscope slides). Glass plates are available prescored, or can be scored with a diamond point and broken into smaller sizes. Disadvantages of breaking the plates include safety hazards and the possibility of producing jagged edges. An advantage of plastic sheets is that they may be cut into various sizes with scissors or a blade.
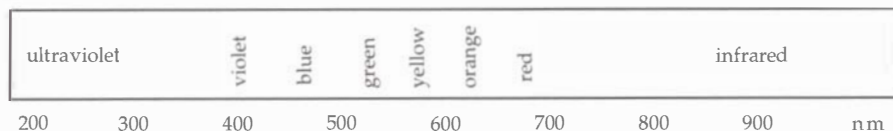
The sorbent layers can be bound or unbound to the glass plate. In the early days of the technique, starches were used as **sorbent binders** in the preparation of TLC plates. Gradually, other binders that gave better results were found. Today the most commonly used binder is gypsum, designated with the letter G. For example, the Merck silica gel G plate has a thin layer of silica gel bound with gypsum.

Sorbent layers can be made from many different materials. The most common sorbent layer used in TLC is silica gel. Many of the modern sorbent layers are based on the modification of silica layers. The -OH functional groups can be replaced with different functional groups, including long chain hydrocarbons. Hydrocarbon functional groups usually make the surface more nonpolar; if this is the case, the sorbent layers can be used for reversed phase chromatography and are designated with the letters RP by the manufacturer. Silica gel layers can also be modified with other functional groups to fine-tune the polarity of the surface. One such sorbent used for polyamide plates is made by the addition of amide groups to the silica gel surface. Another sorbent used in TLC is cellulose. The cellulose fibers used in thin layers are shorter in length than those found in chromatography paper. Sorbent layers can be impregnated with buffers, chelating reagents, metal ions, or many other chemicals to aid in the selectivity and resolution of compounds (Sherma 1991).

A TLC plate may include **chemical additives** that fluoresce upon exposure to ultraviolet light; these additives are present to facilitate the detection of colorless compounds. Fluorescent and phosphorescent substances are excited into an unstable energy state by UV light, and release part of the stored radiant energy when they return to a ground state. This emitted radiation usually lies in the visible part of the spectrum. An illustration of the radiation spectrum is shown in Figure 3.1. When added to the sorbent layer, fluorescent indicators cause the background of the plate to fluoresce or phosphoresce at a given wave-

| ultraviolet | violet | blue | green | yellow | orange | red | infrared |
|---|---|---|---|---|---|---|---|

| 200 | 300 | 400 | 500 | 600 | 700 | 800 | 900 | nm |

length of UV light. Separation zones appear as dark spots on the bright background. Inorganic indicators that phosphoresce upon exposure to short wavelength (centered at 254 nm) UV light include tin-activated strontium compounds, uranyl acetate, magnesium-activated zinc silicate, and zinc cadmium sulfide. Most fluorescent indicators are designated as $F_{254}$ or $UV_{254}$ on the label of the plates.

Conventional TLC and HPTLC plates can be obtained in a variety of sorbent layers. The major differences between TLC and HPTLC plates are the particle size and the particle distribution of the sorbent, and the thickness of the layer. The most common HPTLC sorbent is silica gel; other commercially available plates include cellulose and polyamide. The particle size of silica gel used for HPTLC plates is 5 μm, while that used for conventional plates is 20 μm. The thickness of a high-performance layer is 100–200 μm, compared to 250 μm found on conventional plates. HP layers are more efficient because they produce tighter zones, better resolution, and more sensitive detection.

It should be noted that plates with the same designation from two different manufacturers do not necessarily exhibit the same chromatographic behavior. Plates from different manufacturers may have different layer characteristics even when the same sorbent and binder are used. For example, a precoated silica gel plate from Manufacturer A will not always result in a chromatogram which matches one found on a silica gel plate from Manufacturer B. Once a good separation is obtained on a particular plate, it is important to "standardize" the plate used (Touchstone and Dobbins 1983a).

Two principal plates for the identification of binding media in paint are considered in this publication. The hydrolysates of protein samples are separated on Macherey-Nagel MN300 cellulose plates for the identification of proteinaceous binders, and Merck HPTLC silica gel $F_{254}$ plates are used in the identification of waxes, resins, and sugars.

## Solvent Systems

A TLC solvent system is a liquid mobile phase composed of one or more miscible solvents. The solvent system competes with the dissolved analyte for the active sites on the sorbent and must be carefully selected to achieve a good separation of individual components. Solvent systems are selected by considering the equilibrium between the solvent, the solutes, and the sorbent layer. Often, solvent systems are chosen by trial-and-error methods, or are based on similar applications reported in the literature (Bruno et al. 1989; Stahl 1969). This section presents considerations in the choice of a solvent system, a discussion of the eluting power

of a solvent system, and a summary of some schemes used for solvent system choice.

The selection of a solvent system must take into consideration several factors, the most important being a good separation of the components in the mixture. The choice of the mobile phase depends on the nature of the compounds to be separated. The interactions between the analyte-mobile phase or the analyte-sorbent may be determined by the number and nature of the functional groups in the analyte. A very polar compound will require a mobile phase that interacts strongly with the sorbent layer if the compound is to migrate on the TLC plate. For example, a monosaccharide such as galactose is strongly retained on a silica gel plate and will not migrate in a nonpolar solvent such as benzene. A very polar solvent system incorporating acetonitrile and water will displace galactose from the silica gel plate and promote migration of the sugar. Functional groups of the analyte affect its interaction with the sorbent layer. The retention of an analyte on silica gel increases, in order, with the presence of the following functional groups:

$$RH < ROCH_3 < RN\text{-}(CH_3)_2 < RCO_2CH_3 < RNH_2 < ROH < RCONH_2 < RCO_2H.$$
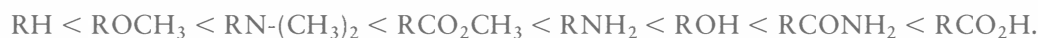
Other factors considered in the selection of a solvent system may include the cost, availability, quality, toxicity, volatility, and miscibility of the solvent or solvents chosen. Simple systems of one or two solvents are preferred over complex mixtures of several solvents. The purest grade solvents should be used since any impurities can greatly affect the selectivity and reproducibility of the separation (Touchstone and Dobbins 1983b). Regulations regarding laboratory safety and waste disposal may limit the choice of solvents that can be used in a conservation lab.

One way of rating the interaction between a particular solvent and a sorbent is based on the eluting power of the solvent, which is defined by the solvent strength parameter, $\epsilon°$. For any given solvent, this parameter represents the adsorption energy per unit area of standard sorbent. A larger $\epsilon°$ indicates a greater interaction between the solvent and the sorbent. In a liquid-solid adsorption process, there is always a competition between solute and solvent molecules for a place on the sorbent surface. The solute molecules will be more readily displaced by a solvent of higher solvent strength. As a result, the $R_f$ value of the solute will increase with an increase in the solvent strength parameter. A solvent that has a high solvent strength parameter on one sorbent, such as silica gel, may have a different solvent strength parameter on a different sorbent. Table 3.1 lists the relative strengths of different solvents on various adsorbents. To prepare TLC mobile phases, solvents from the eluotropic series are blended into binary or ternary mixtures of the correct solvent strength. In most cases, the strength of a solvent mixture will be intermediate between the strengths of the two or more components of the mixture.

| Solvent | $\epsilon°$ | Solvent | $\epsilon°$ |
|---|---|---|---|
| Fluoroalkanes | -0.25 | Methylene chloride | 0.42 |
| n-Pentane | 0.00 | Ethylene dichloride | 0.44 |
| Isooctane | 0.01 | Methyl ethyl ketone | 0.51 |
| Petroleum ether | 0.01 | 1-Nitropropane | 0.53 |
| n-Decane | 0.04 | Triethylamine | 0.54 |
| Cyclohexane | 0.04 | Acetone | 0.56 |
| Cyclopentane | 0.05 | Dioxane | 0.56 |
| 1-Pentene | 0.08 | Tetrahydrofuran | 0.57 |
| Carbon disulfide | 0.15 | Ethyl acetate | 0.58 |
| Carbon tetrachloride | 0.18 | Methyl acetate | 0.60 |
| Xylene | 0.28 | Diethylamine | 0.63 |
| i-Propyl ether | 0.28 | Nitromethane | 0.64 |
| i-Propyl chloride | 0.29 | Acetonitrile | 0.65 |
| Toluene | 0.29 | Pyridine | 0.71 |
| n-Propyl chloride | 0.30 | Dimethyl sulfoxide | 0.75 |
| Benzene | 0.32 | i-Propanol, n-Propanol | 0.82 |
| Ethyl bromide | 0.35 | Ethanol | 0.88 |
| Ethyl sulfide | 0.38 | Methanol | 0.95 |
| Chloroform | 0.40 | Ethylene glycol | 1.1 |

**Table 3.1.**

Solvent strength parameter, $\epsilon°$.

**Advanced Solvent-System Techniques**

Advanced techniques can aid in solvent system selection and optimization of the chromatographic separation.

Computer-aided strategies for mobile phase optimization include using window diagrams, overlapping resolution maps, simplex optimization, and pattern recognition procedures. A structured trial-and-error approach, called the PRISMA model, was developed by Nyiredy for solvent optimization in TLC and HPLC. This model consists of three parts: selection of the chromatographic system, optimization of the selected mobile phase, and selection of the development method (Poole and Poole 1991:1026).

There are trial-and-error methods that can guide the selection of a solvent system for normal phase and reversed phase chromatography. These include spot tests (Bauer et al. 1991) and other simple approaches to solvent selection. Some of these approaches will be summarized here for normal phase chromatography.

The first approach (Hamilton and Hamilton 1987) starts with a solvent of low polarity. Five solvent mixtures are made by adding a more polar solvent in higher proportions. The composition of the more polar solvent in each of the mixtures is 2%, 4%, 8%, 16%, and 32% (by volume). Each increase in the percentage of the more polar solvent corresponds to an 0.05 unit increase in the eluent strength of the solvent system. The unknown sample is tested with these five mixtures. If the resulting $R_f$ is too high, a solvent system with a lower solvent strength is chosen. If the resulting $R_f$ is too low, a solvent system with a higher solvent strength is chosen.

A second approach (Stahly 1993), illustrated in Figure 3.2, starts with testing the unknown sample with dichloromethane as the solvent. If the components travel at the solvent front ($R_f = 1$), the polarity of the solvent system is decreased by adding 25–50% of a less polar solvent, such as hexane. If the components remain at the origin line of the plate ($R_f = 0$), the polarity of the solvent system is increased by adding 0.5–1% of a more polar solvent, such as methanol.
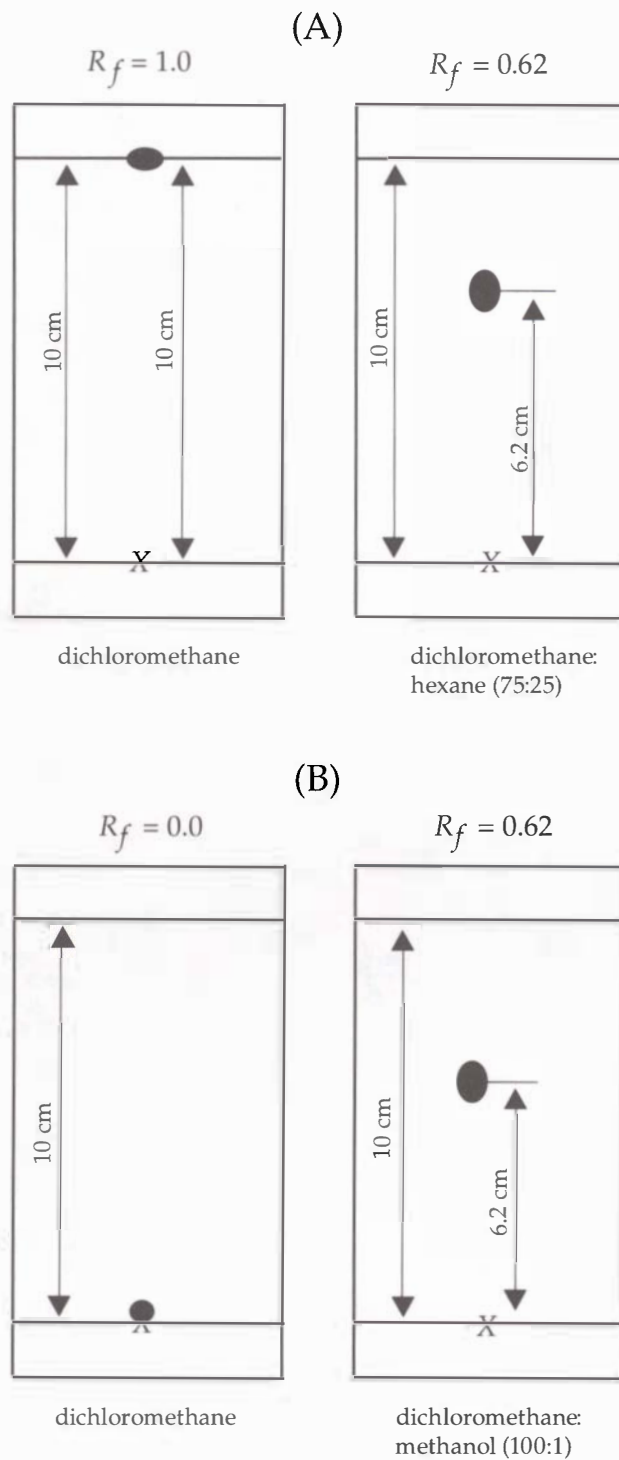
(A)

$R_f = 1.0$                                        $R_f = 0.62$



dichloromethane                          dichloromethane:
                                         hexane (75:25)

(B)

$R_f = 0.0$                                        $R_f = 0.62$



dichloromethane                          dichloromethane:
                                         methanol (100:1)

**Figure 3.2.**

An illustration of a trial-and-error approach to
solvent system development as described by
Stahly (1993).

## Development Chambers

The samples to be separated by TLC are spotted on an origin line near
one end of the chromatographic plate. The plate is placed in contact with
the solvent system, usually inside a development chamber. This section
presents the different modes and apparatus for development.

## Nonlinear Methods of Development

Nonlinear methods, which include **circular** and anticircular development, use horizontal development chambers. Circular development is very similar to the early drop chromatography method described by Izmailov and Schraiber (see Chapter 1, page 10). In this mode of development, the sample or samples are placed in a circle around the center of the plate. Then the plate is placed over a petri dish or special chamber filled with the mobile phase. The solvent system is delivered to the center of the TLC plate by a wick. It flows in an outward direction toward the periphery of the plate. In **anticircular** development, the sample is applied along the outer circumference and developed toward the center of the plate. Figure 3.5 shows a diagram of chromatograms resulting from circular and anticircular development methods. The circular development mode is advantageous for separation of components with low $R_f$ values, and anticircular development is advantageous for the separation of components with high $R_f$ values.

Optimal HPTLC circular and anticircular development can be performed in specially designed U-chambers. One horizontal type of chamber electronically controls the mobile phase velocity using a stepping motor. This motor drives a syringe that feeds the mobile phase directly to the center of the horizontal plate. A similar chamber designed for anticircular development feeds the solvent to the outer circumference of the plate (Poole and Poole 1991).

Advanced techniques include two-dimensional, multiple, and continuous development, as well as forced flow techniques, such as overpressured chromatography and centrifugal chromatography.

The many development techniques in TLC include linear, circular, anticircular, multiple, continuous, and two-dimensional. Figure 3.3 illustrates the relationship between these development techniques. The most typical approach is to use a linear development chamber with the mobile phase moving vertically up the plate.

Linear chambers are usually rectangular in shape, though cylindrical chambers can also be used. This type of chamber may be as simple as a jar with a tightly sealed lid, and may be used in a saturated or an unsaturated state. Saturation of a development chamber is achieved by lining the chamber with filter paper or a saturation pad and pouring the solvent system over the paper. Then the chamber is covered and set aside to allow vapor equilibration. Enough solvent is placed in the chamber so it covers the bottom of the chamber but remains below the point of application of the sample to the TLC plate. Once the tank has been equilibrated (usually 15–30 minutes), the tank is quickly opened, the plate is inserted, and the tank is again covered. In the case of an unsaturated chamber, the solvent is poured directly into the empty chamber, the plate is inserted, and the chamber is quickly covered.

A second type of linear development chamber is the sandwich or S chamber (see Figure 3.4). This chamber is composed of a backing plate (metal or glass), a trough for the solvent system, and clips. The sandwich chamber is often used with HPTLC plates and uses a much smaller volume of the solvent system than the conventional TLC chamber. The chromatographic plate is attached to the backing plate so there is minimal vapor space between the plates. It is then placed in the trough of the sandwich chamber, which holds about 5 ml of the solvent system. Since the amount of space between the plate and the surrounding vapor is minimized, there is less solvent evaporation from the surface of the chromatographic plate; thus, less solvent is used. One disadvantage of the sandwich system is that highly volatile solvents systems may
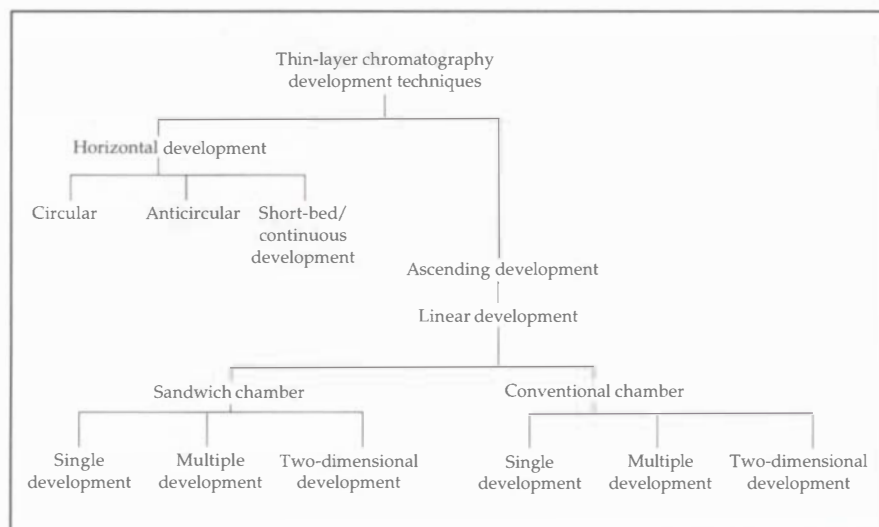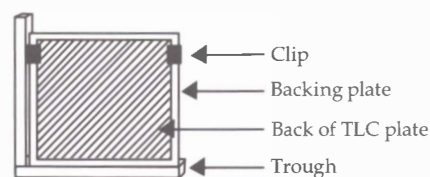


**Figure 3.3.**

A general classification scheme of chromatographic development techniques.

**Figure 3.4.**
A schematic diagram of the components of a
sandwich chamber, including the backing plate,
clips, TLC plate, and trough.

evaporate from the chamber before the development is complete.
Also, the solvent front may become uneven as a result of preferential
evaporation at the edges of the plate caused by air currents. To decrease
the evaporation of the volatile solvents and to keep the vapor space
saturated, the entire sandwich chamber is placed inside a tightly sealed
vessel.

Plates can also be developed with the solvent system
*descending* through the TLC plate. This method was first used in paper
chromatography. The solvent is fed to the top of the development
chamber through a wick arrangement. This is a more cumbersome, rarely
used method of linear development which shows no significant advan-
tages over ascending methods (Fried and Sherma 1986).

**Two-dimensional development** is used for the separation of
complex mixtures and allows for the use of solvents with different selec-
tivities. The sample is applied to one corner of the TLC plate, which is
developed in a linear development tank with the mobile phase ascending
through it. The plate is removed and allowed to dry. The plate is then
rotated 90° and redeveloped in a different solvent. The components of the
sample are separated over the surface of the entire plate. Disadvantages
of this method include difficult interpretation and reduced reproduc-
ibility. Quantitation is not possible for two-dimensional TLC because
standards can be applied only after the first development and will not
have the same zone configuration as the doubly developed zones.

**Multiple development** can be performed manually or with
automated equipment. The manual technique involves repeated develop-
ments of the TLC plate in the same solvent and in the same direction. The
plate is first developed normally in a linear chamber with the solvent
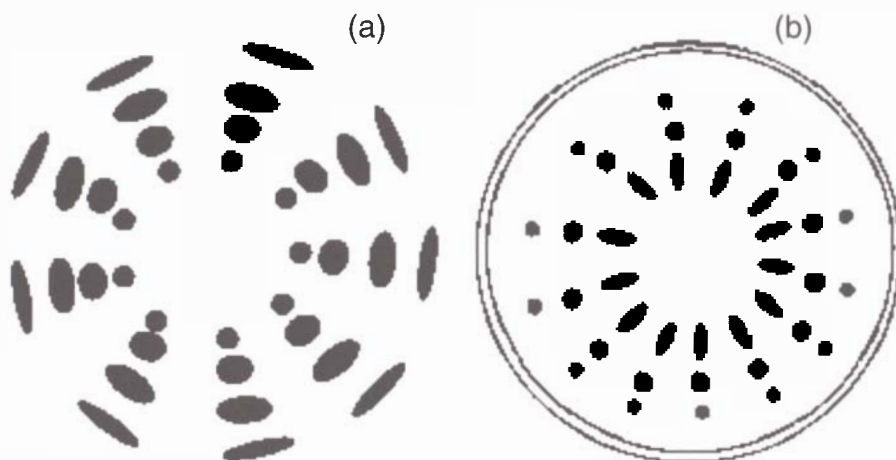


**Figure 3.5.**
Circular development with (a) the point of
solvent entry at the plate center, and (b) anti-
circular development from the outer circle
toward the center.

**Additional Development Techniques**

An automated instrumental method of multiple development (AMD), designed by the Swiss company CAMAG, allows a TLC plate to be repeatedly developed in the same direction over increasing migration distances (Jaenchen 1991). Typical distance increments are 3 mm or less for a 20–25-step development. Unlike the manual method described above, the mobile phase for AMD typically differs with each successive development. AMD uses a solvent gradient starting with a very polar solvent, through a solvent of medium polarity, and ending with a very nonpolar solvent. The method is suitable for the separation of complex samples that differ widely in the polarity of their components. A disadvantage of this technique is the expense of the AMD instrument.

The method of **continuous development** uses a chamber similar to the horizontal chamber, which allows the plate to protrude from the top of the tank so that the solvent continuously evaporates. The short-bed, continuous-development chamber (SB/CD chamber) is illustrated in Figure 3.6. It has a low profile and a wide base to permit development close to the horizontal position. The TLC plate is positioned so that the bottom edge is in contact with the solvent and the top edge is resting on the back wall of the chamber. A glass plate cover is positioned over the chamber and touches the upper edge of the TLC plate. In this configuration, the solvent evaporates from the junction of the TLC plate and the cover plate. This method effectively lengthens the plate to improve resolution and is excellent for the separation of complex mixtures with components that have low $R_f$ values. If conditions are optimized, continuous development results in faster separations than those obtained with conventional development (Nurok et al. 1982).
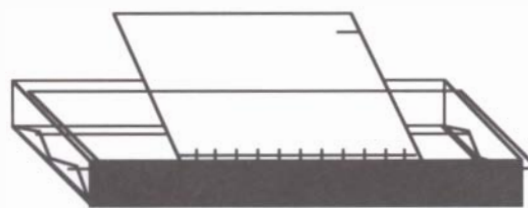


**Figure 3.6.**

A schematic diagram of a short-bed, continuous-development chamber. The TLC plate is placed inside the chamber and rests on its back wall. A glass plate covers the chamber and rests against the TLC plate.

ascending through the plate. The plate is then removed from the chamber and air dried for 5 to 10 minutes. Next, the plate is placed in the same solvent system and the development is repeated. This process, which can be repeated numerous times, increases the resolution of components with $R_f$ values below 0.5. Repeated movement of the solvent front through the spots from the rear causes spot reconcentration and compression in the direction of the multiple developments. The broadening of the spots, usually seen during chromatographic migration, is reduced with multiple development. The technique can also be carried out with different solvents in the same direction.

## Detection Methods

Once the TLC plate has been developed, it is removed from the chamber and air dried to remove the mobile phase. The plate is then closely scrutinized to identify the separation zones. Colored substances may be viewed in daylight without any treatment. If the substances are colorless, other techniques must be employed to detect the zones. These include both destructive methods, where the compound or compounds are permanently changed, and nondestructive methods, where the compound is not altered by the visualizing technique. This section takes a cursory look at detection reagents and describes the basic apparatus needed for postchromatographic detection. A more detailed treatment of detection methods is given in Chapter 9.

An ideal visualization or location procedure for thin-layer chromatography should be able to:

1. reveal microgram quantities of separated substances;
2. yield distinct spots;
3. show a satisfactory contrast between the visualized area and the background; and
4. provide a visualized area that is stable or suitable enough for a quantitative measurement, if desired (Touchstone and Dobbins 1983c).

There are two methods of visualization: physical and chemical. The use of ultraviolet light to observe fluorescing zones is an example of a

One of the newest techniques for high-performance TLC is forced flow or **overpressured layer chromatography** (OPLC). In this instrumental technique, the TLC plate is placed inside a chamber and covered by a flexible plastic membrane that is held close to the surface by pressurized gas. The mobile phase is forced through the sorbent layer by a pump operating at about 1 MP (Sherma 1991). The flow velocity of the mobile phase is controlled in OPLC. The technique can be used for linear, circular, or anticircular development. Over-pressured chromatography provides a means of rapid development with constant and adjustable flow rates of the mobile phase. The method can be used for continuous and gradient development, and prevents the diffusion of separation zones. Overpressured chromatography uses a low volume of solvent and provides reproducible $R_f$ values. New trends in OPLC include high-pressure versions similar to HPLC, temperature programmed OPLC, on-line one-dimensional OPLC, and two-dimensional OPLC. The main disadvantage of the technique is the added instrumentation, which leads to a higher cost than conventional TLC (Ferenczi-Fodor et al. 1991).

physical method, while the reaction of sulfuric acid with the zones to produce a brown or charred area is an example of a chemical one.

The observation of fluorescence or phosphorescence on a TLC plate requires an ultraviolet light source. A portable laboratory lamp with short wave and long wave output is useful for examination of TLC plates. Sophisticated cabinets are available for viewing the TLC plate under ultraviolet or visible light. An alternative, inexpensive viewing and photographic box can be made in the laboratory (see Chapter 8, pages 74–75; and Protocol G).

Chemical methods of detection include prechromatographic and postchromatographic derivatization. The latter usually requires a visualization reagent that is placed in contact with the substances to be detected on the plate. The two procedures for applying the visualization reagent are spraying and dipping. Spray guns using a gas propellant or a glass atomizer are apparatus typically used for spraying the visualization reagents. Dipping can be performed in a normal rectangular TLC developing chamber. The large volume of the developing chamber may be a drawback when using visualizing reagents of high toxicity. Small volume tanks made specifically for dipping TLC plates are commercially available. CAMAG manufactures a Chromatogram Immersion Device for HPTLC plates up to $20 \times 10$ cm in size. This holding time in the reagent can be preselected for between 1 and 10 seconds for accurate control of the dipping procedure. Once the visualization reagent is applied, it is often necessary to heat the plate. This can be done in a laboratory oven or hot plate heating apparatus designed for heating TLC plates uniformly.

## Documentation

The documentation of a thin-layer chromatogram may be as simple as an annotated sketch or tracing of the plate. A photocopy of the plate may be useful if the separation zones are seen as distinct colored spots, but may be limited in the ability to show weak solutes or certain colors. Laboratory photography has become the method of choice for documentation of TLC plates because of its ease, durability, and accuracy. TLC plates can be photographed with 35-mm camera equipment or instant image photography. Integrated camera and lighting systems are now on the market. A detailed presentation of documentation procedures for TLC is given in the literature (Vitek 1991) and is discussed in Chapter 8 herein.

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Chapter 4

**Analysis of Proteins by Thin-Layer Chromatography**

The purpose of this chapter is to present:

- A summary of the history and chemistry of proteinaceous materials as it pertains to artists' materials
- A description of the methodology, sample preparation, and technique for using TLC to analyze proteinaceous binders, coatings, and adhesives
- An example of the application of the technique to an actual protein sample from an actual artifact

**A Summary of the Use and Chemistry of Proteinaceous Binders**

This section gives a brief summary of the types of proteins that may be found in paintings or objects. Detailed information on the uses of proteins in medieval painting (Thompson 1956) and a description of modern uses of proteins can be found in other sources (Mayer 1991). An excellent description of the chemistry of proteins used in the fabrication of museum objects is given in chapter 7 of *The Organic Chemistry of Museum Objects* (Mills and White 1986).

Proteins are a class of materials used as binders in paintings throughout history. This class includes glues, egg (egg white, egg yolk, or whole egg), and casein. Glues made from animal or fish skin, bone, and other parts have been used as binders or adhesives in the sizing of paper, and in the ground of paintings. Egg white has been used in the illumination of manuscripts, as the adhesive for gold leaf, and as a temporary varnish on paintings. The traditional binder in tempera paints is egg yolk. Casein, a milk by-product, has been used in wall paintings and panel paintings, painted furniture and painted textiles, and theatrical stage sets (Kuhn 1986).

Proteins are high molecular weight organic molecules that are composed of a sequence of amino acids that are specific to each protein. There are twenty-two naturally occurring amino acids that may be found in the chemical framework of a protein. The amino acids are bound to each other by peptide linkages (-CO-NH-). The molecular weight of proteins ranges from $10^4$ to $10^7$ (Mills and White 1986).

## Analytical Methodology

Identification of proteins by thin-layer chromatography is based on the presence or absence of certain amino acids within the protein. First, the protein in the sample is hydrolyzed, or broken down, into its component amino acids. Next, the sample, individual amino acids, and reference materials are analyzed by conventional thin-layer chromatography on a cellulose stationary phase. A butanol, acetic acid, and water solvent system is used. The amino acids are visualized on the TLC plate using a ninhydrin detection reagent. The presence of hydroxyproline and large quantities of proline, seen as yellow spots in the chromatogram, indicate the presence of gelatin found in animal glues. Further differentiation of protein binders is based on the amount of particular amino acids present in the sample.

## Sample Preparation

Sample preparation for the analysis of proteins requires the preparation of three types of samples, including unknown samples, reference samples, and amino acid samples. The unknown and reference samples contain large protein molecules that must be broken down into their component amino acids by acid hydrolysis before analysis. Amino acid samples are prepared from a commercially available set of pure amino acids.

Any identification strategy for proteins must take into account possible losses of certain amino acids from acid hydrolysis. Large amounts of the amino acids tyrosine and tryptophan can be destroyed by acid hydrolysis. Other amino acids, such as serine and threonine, may also be affected (Brenner et al. 1965).

Unknown samples for protein identification and well-characterized reference materials are hydrolyzed using high-purity $6N$ hydrochloric acid. Samples should be 0.5–0.7 mg in size. Reference samples can include commercially available compounds such as casein, rabbit-skin glue, egg white, egg yolk, whole egg, and fish glue. Liquid phase or vapor phase acid hydrolysis can be performed under vacuum. The procedure for acid hydrolysis can be performed in equipment assembled from other laboratory supplies or in specially designed apparatus, such as the Pierce Reacti-Therm sample incubator with hydrolysis tubes.

One procedure for vapor phase hydrolysis uses simple laboratory supplies and follows a series of steps. The vapor phase reacts with the samples to give a high yield of the amino acids. This method has the advantage of needing only a very small amount of acid, and up to fourteen samples can be hydrolyzed at one time. It works best with protein samples. Equipment needed includes a reaction chamber, sample vials, a vacuum pump, a tank of high-purity nitrogen, and a laboratory oven. The reaction chamber is assembled from a large Pierce vial that is fitted with a Miniert valve and a septum attached to a screw-top lid. (Note: A new septum must be used for each hydrolysis to ensure a good seal.)

The procedure for vapor phase acid hydrolysis follows seven basic steps:

1. The samples are weighed directly into labeled 1-ml glass vials and then placed inside the reaction chamber.
2. Next, 200 µl of constant boiling 6N hydrochloric acid (Pierce, sequanal grade) is added to the bottom of the reaction chamber.
3. The lid is tightly screwed on to the chamber.
4. The assembly is evacuated using a small vacuum pump. To do this, a large syringe needle is attached to vacuum tubing that in turn is attached to the pump. The needle is introduced into the reaction chamber through the Miniert valve, which has been placed in the open position. The vacuum pump is turned on and the chamber is evacuated.
5. The chamber is flushed with nitrogen, which again is introduced into the chamber by placing a needle through the open Miniert valve. The needle is attached to tubing and a regulator of a high-purity nitrogen gas tank. Three cycles of evacuation and nitrogen flushing are done to remove residual oxygen, ending with a final evacuation of the reaction chamber.
6. The reaction chamber is placed inside a preheated laboratory oven and heated at 105 °C for 24 hours.
7. The chamber is removed from the oven, allowed to cool for 10 minutes, and opened. The sample vials are removed, and the samples are evaporated to dryness under a stream of nitrogen. The samples are reconstituted in 400 µl of 0.1N HCl. The sample vials are capped and placed in a refrigerator until use.

Solutions of individual amino acids are prepared and used as controls for the comparision of $R_f$ values. These solutions are made by dissolving 1 mg of high-purity amino acid in 1 ml of 0.1N HCl. The solutions are stored in tightly sealed glass vials and can be kept in the refrigerator for 2–4 weeks.

## Technique

Hydrolysates of proteins have been separated on both silica gel and cellulose plates (Brenner et al. 1965). A review of the literature indicates that cellulose, a highly polymerized polysaccharide, provides advantages over other stationary phases for separation of amino acids (Bhushan 1991). Cellulose plates (Macherey-Nagel MN300 20 × 20 cm) were used during the Getty Conservation Institute TLC course for the analysis of proteinaceous binders.

The cellulose plate is washed in reagent grade methanol 24 hours before analysis. Approximately 30 ml of methanol are placed in

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### An Alternative Method for Liquid Phase Acid Hydrolysis

Another procedure for liquid phase acid hydrolysis uses a sample heating unit (Pierce Reacti-Therm, Model no. 18800), 1.0-ml vacuum hydrolysis tubes (Pierce, Model no. 29550), an aluminum heating block (Pierce Reacti-Block H), and a gas manifold fitted with Teflon-coated needles for evaporation of samples (Pierce Reacti-Vap, Model no. 18780). Up to nine samples may be prepared simultaneously. The samples are weighed directly into 1-ml vacuum hydrolysis tubes. A 150-ml volume of constant boiling 6N hydrochloric acid (Pierce, sequanal grade) is added to each sample. Each tube is placed in an aluminum heating block set in a sample heating unit. The tubes are loosely capped with a Teflon stopper. Tubing is used to connect the sidearm of each hydrolysis tube to a portable vacuum pump. Air is evacuated using the pump, and the tube is tightly sealed. The samples are heated at 110 °C for 18–72 hours, then cooled to room temperature before opening. The gas manifold is fitted to the hydrolysis tubes so that the Teflon-coated needles are placed just above each sample. The samples are evaporated to dryness under a stream of nitrogen, then reconstituted in 400 µl of 0.1N HCl. The solutions are transferred with Pasteur pipettes to 1-ml vials.

a conventional TLC chamber. The plate is placed in the chamber and developed completely to remove any organic impurities on the plate. The plate is dried in the fume hood and stored in a desiccator until its use.

Several solvent systems have been reported for the separation of amino acids. Of these systems, the n-butanol : glacial acetic acid : water system (80:20:20) has proven to be most successful for separating amino acids on the Macherey-Nagel cellulose plate. For development of a TLC plate spotted with unknown, reference, and amino acid samples, 30 ml of this solvent system are prepared and placed in a clean, dry, conventional chromatography chamber. A piece of TLC Whatman paper is placed in the solvent inside the chamber to act as a saturation pad. The chamber is tilted until the Whatman paper is completely wet with the solvent system. The chamber is covered with a glass lid and preequilibrated in the solvent system for 30 to 45 minutes.

A developed plate is dried at room temperature in a fume hood for about 30 minutes, then evenly sprayed with a ninhydrin reagent prepared as a 2% (w/w) solution in ethanol. After spraying, the plate is dried, then heated at 100 °C for 10 minutes in an oven.

The plate is evaluated and photographed 24 hours after spraying with the detection reagent to ensure complete development and visualization of the colored spots. The documentation of TLC plates is presented in Chapter 8.

## Applications

A sample from a fourth-century C.E. Romano-Egyptian sarcophagus (J. Paul Getty Museum, 82.AP.75) shown in Figure 4.1 was analyzed by TLC.

Proteinaceous binders were analyzed and identified by the presence or absence of amino acids in the chromatogram. Samples included six amino acid reference solutions, six hydrolyzed reference proteins, and a hydrolyzed sample taken from the sarcophagus. In addition, two pigment-binder mixtures, made using proteinaceous binders, were analyzed to examine possible interferences by pigments. Figure 4.2 shows the thin-layer chromatogram for this analysis.

This chromatogram has seventeen lanes containing a series of spots or patterns for each sample. Most spots are colored violet to purple. The spot for hydroxyproline is colored yellow, making it more difficult to see. The six most important amino acids for identification of proteinaceous binders are spotted in the first six lanes of the plate: glutamic acid, hydroxyproline, lysine, serine, threonine, and tyrosine.

Lanes 14–16 contain hydrolyzed pigment-binder mixtures. The sample in lane 14 is a mixture of rabbit-skin glue and vine black pigment. The sample in lane 15 is a mixture of whole egg and lead white pigment, and lane 16 contains the sample of an egg yolk and lead white mixture. The presence of the pigment in these samples does affect the

**Figure 4.1.**

A photograph of the fourth-century c.e. Romano-Egyptian sarcophagus (J. Paul Getty Museum, 82.AP.75). A sample was taken from a red pigmented area for protein analysis by TLC.

retention of the components, seen as slight tailing of the spots, but does not limit the ability to identify the material.

As seen in Figure 4.2, animal glues such as rabbit-skin glue and fish glue contain relatively large quantities of hydroxyproline, while other materials (i.e., egg and milk products) do not. Thus, animal glues can be identified by the presence of hydroxyproline. However, different types of animal glues cannot be distinguished by the chromatographic pattern. Casein may be identified by the presence of higher proline concentrations and the absence of hydroxyproline. *(This identification can be tentative due to the variety of ways in which casein is processed and used. Such an identification should be confirmed using another method.)* Other distinctions in the chromatographic patterns are not readily obvious by eye. A computer method for identification based on a scanned digital image and statistics is currently under development. (See Chapter 8, page 76, for additional details.)

A red pigmented sample from the sarcophagus was analyzed for the presence of a protein binder. Located in lane 17, the sarcophagus

**Figure 4.2.**

TLC analysis of amino acids, hydrolyzed reference proteins, and unknown sample taken from a Romano-Egyptian sarcophagus. Stationary phase: Macherey-Nagel MN300 cellulose. Mobile phase: butanol : acetic acid : water (80:20:20). Detection reagent: 0.2% ninhydrin in ethanol. Lanes: (1) glutamic acid, (2) hydroxyproline, (3) lysine, (4) serine, (5) threonine, (6) tyrosine, (7) Sigma standard, (8) whole egg, (9) egg white, (10) egg yolk, (11) casein, (12) rabbit-skin glue, (13) fish glue, (14) rabbit-skin glue + vine black pigment, (15) whole egg + lead white pigment, (16) egg yolk + lead white pigment, and (17) sarcophagus sample.
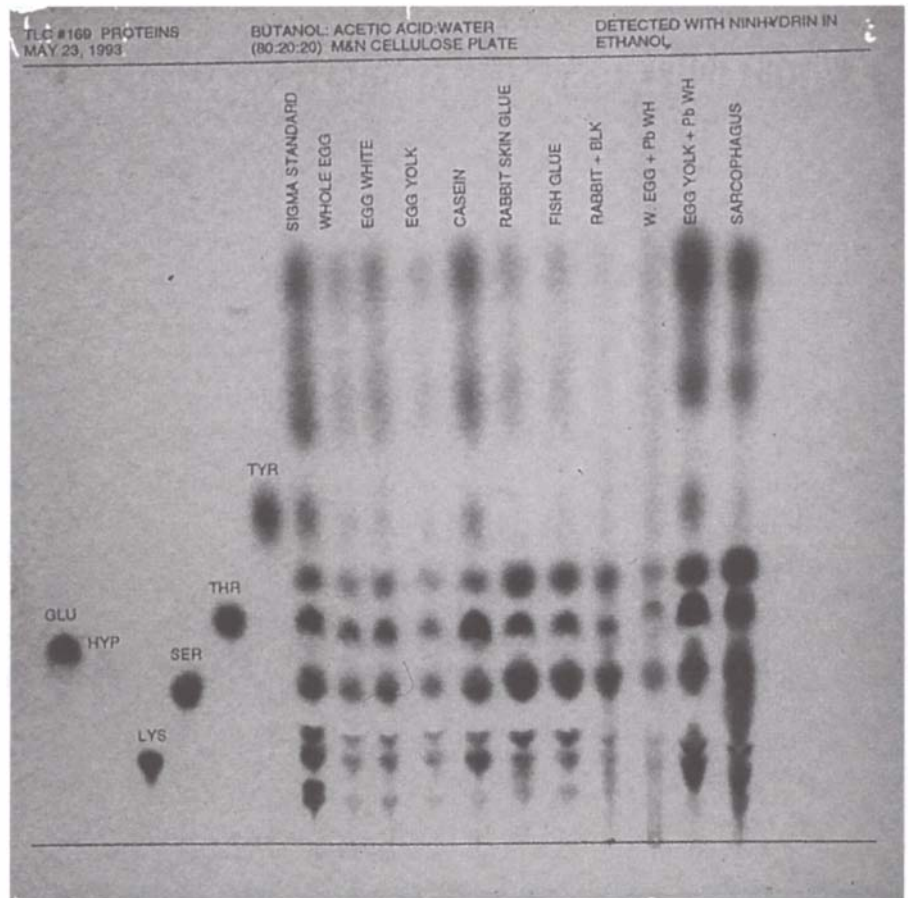
sample is highly concentrated and near the loading limit of the plate. Its chromatographic pattern is very similar to that of a hide glue (rabbit-skin glue reference). In addition, it contains large quantities of both proline and hydroxyproline. Thus, the sample is identified as an animal glue. This result was confirmed by gas chromatography.

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# Chapter 5

## Analysis of Carbohydrates by Thin-Layer Chromatography

The purpose of this chapter is to present:

- A summary of the history and chemistry of carbohydrates or gums as it pertains to artists' materials
- A description of the methodology, sample preparation, and technique for using TLC to analyze gum binders, coatings, and adhesives
- An example of the application of the technique to a gum sample from an actual artifact

### A Summary of the Use and Chemistry of Carbohydrate Binders

Chapter 6 of *The Organic Chemistry of Museum Objects* (Mills and White 1986) is recommended reading for an understanding of the chemistry and analysis of carbohydrates.

Plant gums and other carbohydrates are used as paint binders, adhesives, and additives in water-soluble artists' materials. Historically, plant gums have been used as binders in Egyptian wall paintings. Later they were used as the paint binders in illuminated manuscripts. Gums such as gum arabic are found in watercolor and gouache paints, and gum tragacanth is used as a binder for pastel crayons. Before the modern practice of adding small amounts of glycerol to aqueous paint media, simple sugars like honey were sometimes used to promote elasticity in watercolors. Starch, another carbohydrate, is often used to make adhesive or size.

Plant gums are carbohydrates, a class of chemicals that occur in natural products and are often exuded by plants. **Monosaccharides** are the chemical building blocks of carbohydrates. If monosaccharides are found singly as monomers, they are considered to be simple sugars. If they are chemically bound together in a polymeric network, they are considered to be complex sugars, also called **polysaccharides**. Both simple and complex sugars are used as binding media or as binding media additives. Honey consists mainly of sucrose—a disaccharide—and variable amounts of the monosaccharides glucose and fructose. Gum arabic, on the other hand, is a complex sugar with a polymeric structure containing large amounts of arabinose and

galactose; rhamnose, glucuronic acid, and galacturonic acid are minor components.

## Analytical Methodology

Carbohydrates are identified by the presence or absence of certain monosaccharides within the carbohydrate. To prepare the binding media sample for TLC, the carbohydrate is broken into monosaccharides by hydrolysis with dilute hydrochloric acid. Hydrolyzed samples, monosaccharide solutions, and hydrolyzed reference materials are analyzed on an HPTLC silica gel plate. The plate is developed in an acetonitrile : water solvent system in a sandwich chamber. The individual sugars are visualized by spraying the developed plate with an aminohippuric acid detection reagent. After heating the plate, it is examined under ultraviolet light. A variety of fluorescent colors for the different monosaccharides are observed, ranging from pink (arabinose) to orange (glucuronic acid).

## Sample Preparation

The samples needed for this carbohydrate analysis include samples of the unidentified binding medium, reference carbohydrates (gum arabic, cherry gum, etc.), and monosaccharide solutions (arabinose, rhamnose, galactose, etc.). Unknown samples and reference carbohydrates are hydrolyzed using a dilute concentration of hydrochloric acid. The hydrolysis of carbohydrates can be difficult, as it can result in a considerable amount of humin formation if carried out in the presence of large amounts of inorganic material.

The samples are hydrolyzed in a Pierce vial fitted with a Miniert valve using liquid phase hydrolysis methods. Each sample is weighed directly into a 1-ml vial. Four hundred $\mu$l of $0.3N$ HCl are added to each sample. The uncapped glass vials are placed in the Pierce vial. Air is evacuated from the reaction vial using a portable vacuum pump. The valve is opened and the interior of the vial is flushed with high-purity nitrogen gas. The cycle of evacuating and flushing with nitrogen is repeated three times. The assembly is placed in a laboratory oven and heated to $95 \pm 5$ °C for 5 hours. The Miniert valve is opened about 10 minutes after the Pierce vial is removed from the oven. The Pierce vial is then opened and the sample vials are quickly capped and refrigerated until analysis.

Solutions of arabinose, fucose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, rhamnose, ribose, and xylose are prepared in a 1-mg/ml concentration from commercially available monosaccharides. Approximately 2 mg of each monosaccharide is dissolved in 2 ml of HPLC grade methanol to obtain this concentration.

### Technique

**HPTLC silica gel plates** (Merck silica gel 60 $F_{254}$) are used in the analysis of carbohydrates. The plates are first washed in methanol, then activated at 100 °C for 1 hour. After cooling, they are stored in a desiccator. The plates are activated within 24 hours of development. Glass micropipettes are used to spot sample solutions on a baseline 1 cm from the bottom of the plate. The chromatogram is developed in an acetonitrile : water (85:15) **solvent system,** using a sandwich chamber. Five ml of the solvent system are added to the trough of a sandwich chamber. The chromatographic plate is attached to the backing plate with clamps and inserted into the trough. The entire unit is placed inside a covered presaturated cylindrical chamber to prevent air drafts that can affect the travel of the solvent front (see Figure 5.1). The plate is developed to a distance of 8 cm, which usually takes 20 minutes.

After development, the plate is dried in the fume hood. Then it is sprayed with an **aminohippuric acid** detection reagent: a 0.3% solution of aminohippuric acid with 3% phthalic acid in ethanol. This solution is made by dissolving 0.3 **g** of 4-aminohippuric acid and 3 g of phthalic acid in 100 ml of ethanol. The solution, which is stable for several days, is sprayed onto the plate in a homogeneous coating. It is likely that the sugars react with the reagent to form Schiff's bases. The plate is dried in the fume hood before being placed in an oven and heated at 100 °C for 10 minutes. When viewed under long-wavelength UV light, the separated zones fluoresce (Jork et al. 1990).
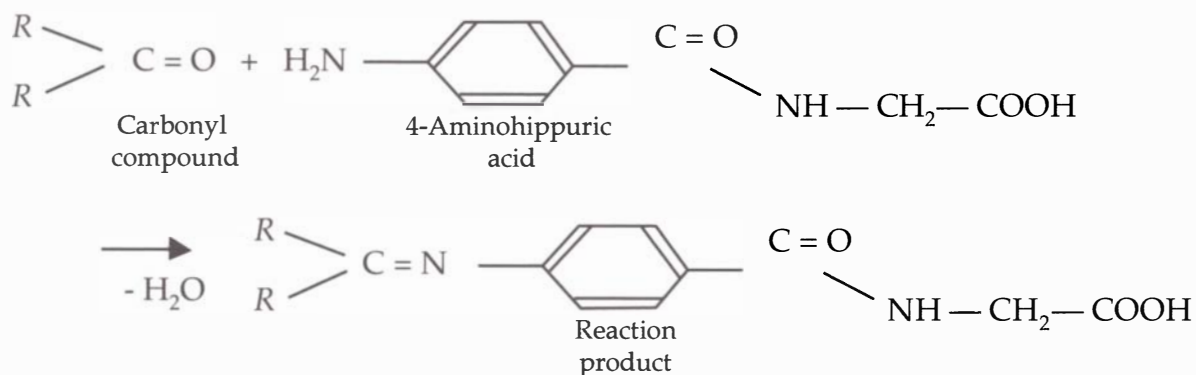


The plate is evaluated, then photographed under ultraviolet light (366 nm), using both black-and-white print and color slide film. The documentation of the TLC plate is described in further detail in Chapter 8 of this book.

### Applications

An 800-μg sample of a clear, resinous material was taken from *Inlet*, a Robert Rauschenberg painting executed in 1959 (Museum of Contem-

**Figure 5.1.**

A schematic diagram showing the placement of a sandwich chamber inside a cylindrical chamber to minimize the effect of air currents.



Lid

Cylindrical chamber

Sandwich chamber assembly
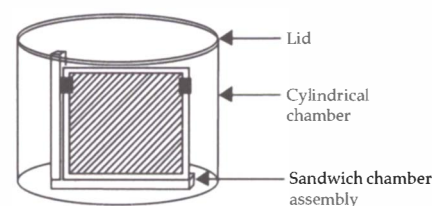
porary Art, Los Angeles). The sample was hydrolyzed according to procedures described above. The sample was then spotted onto an activated silica gel plate at three different volumes: 0.2, 0.5, and 1.0 μl. Monosaccharide and reference solutions were spotted at a 0.5-μl volume.

Figure 5.2 shows the thin-layer chromatogram for this carbohydrate analysis. The chromatographic system allows complete separation of galacturonic acid, glucuronic acid, galactose, arabinose, and rhamnose. Other simple sugars, such as xylose, fucose, and ribose (not in mixture), or glucose and mannose, are not completely resolved. The ascending order of $R_f$ values for sugars is: galacturonic acid $<$ glucuronic acid $<$ galactose $<$ glucose $\approx$ mannose $<$ arabinose $<$ fucose $\approx$ xylose $\approx$ ribose $<$ rhamnose.

All reference gum samples (e.g., gum tragacanth, gum arabic, etc.) analyzed on this plate show distinguishable chromatographic patterns. However, dextrin and wheat starch cannot be differentiated on the basis of their chromatograms.

**Figure 5.2.**

TLC analysis of simple sugars, hydrolyzed reference carbohydrates, and unknown sample taken from Rauschenberg's *Inlet* (spotted at several volumes). Stationary phase: Merck HPTLC silica gel 60 F$_{264}$. Mobile phase: acetonitrile : water (85:15). Detection reagent: 0.3% aminohippuric acid + 3.0% phthalic acid in ethanol. Lanes: (1) rhamnose, (2) mannose, (3) ribose, (4) Rauschenberg sample, (5) galacturonic acid, (6) fucose, (7) glucuronic acid, (8) Rauschenberg sample, (9) arabinose, (10) xylose, (11) glucose, (12) Rauschenberg sample, (13) galactose, (14) gum arabic, (15) gum tragacanth, (16) cherry gum, (17) dextrin, (18) wheat starch, and (19) guar gum.
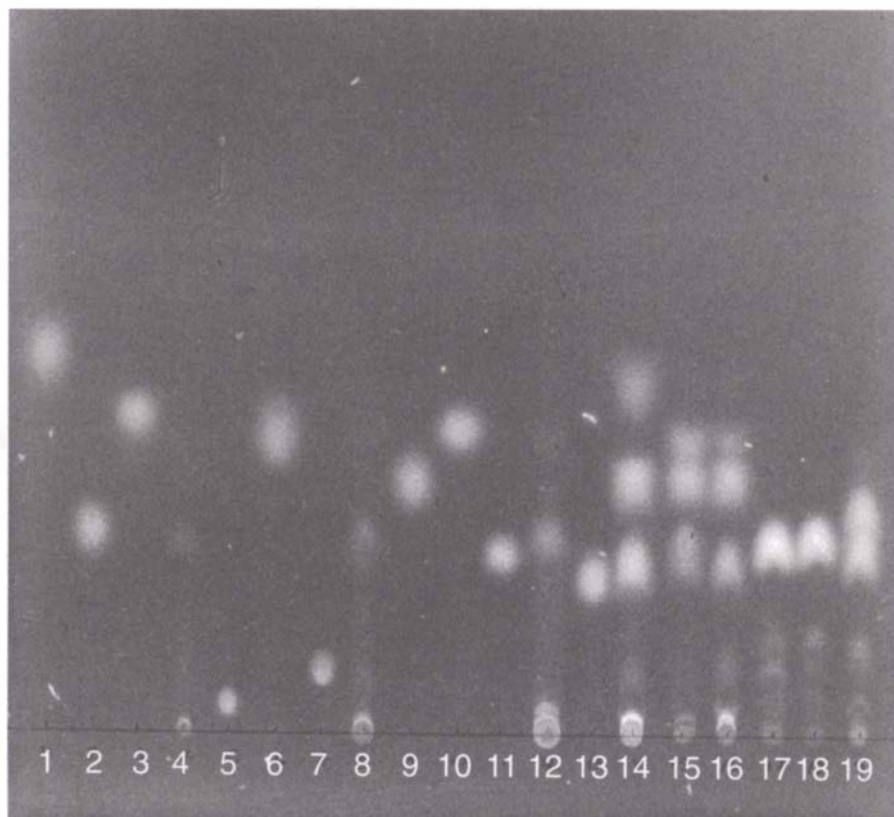
The sample from the Rauschenberg painting was spotted in lanes 4, 8, and 12, in volumes of 0.2, 0.5, and 1.0 $\mu$l, respectively. The pattern of spots for this sample most closely matches those of the starch and dextrin reference solutions. Glucose, with an $R_f$ value of 0.275, is the most prominent monosaccharide in these three samples. Lane 12, the lane with the largest volume of the binding media sample on the plate, shows a faint orange spot with an $R_f$ value of 0.406. This spot is characteristic of xylose or ribose. The sample from the Rauschenberg painting may be an adhesive made from dextrin or starch with a plasticizer added (Fishman 1986). This result is confirmed by FT-IR microscopy, where the infrared spectrum of the Rauschenberg sample most closely resembles a dextrin spectrum.

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# Chapter 6

## Analysis of Waxes by Thin-Layer Chromatography

The purpose of this chapter is to present:

- A summary of the history and chemistry of waxes as it pertains to artists' materials
- A description of the methodology, sample preparation, and technique for using TLC to analyze wax binders, coatings, and adhesives
- An example of the application of the technique to a wax sample from an actual artifact

### A Summary of the Use and Chemistry of Wax Binders

Chapter 4 of *The Organic Chemistry of Museum Objects* (Mills and White 1986) presents the chemistry and analysis of waxes and is recommended reading. Waxes are generally considered to be low-melting translucent solids with a "waxy" feel.

Waxes were used by the Egyptians as waterproofing materials, adhesives, and paint binders. The encaustic technique involves the suspension of pigments in a wax binder to create a painting. Waxes have been utilized for candle making since the Roman era, and since medieval times as a component of document seals. They are also used in metal casting techniques, and in such conservation practices as the relining of paintings.

Waxes are chemically complex materials that contain long-chain hydrocarbons, acids, alcohols, and esters. In addition, they may contain plant sterols, triterpenoids, and their esters. Waxes are products originating from several sources, including animals, insects, plants, and minerals. Insect and animal waxes include beeswax, spermaceti wax, and lanolin. Plant waxes include carnauba wax, candelilla, and Japan wax. Other waxes are associated with fossilized materials, such as ceresine wax, earth wax, and paraffin (a petroleum product).

### Analytical Methodology

A phenomenological approach is taken for the identification of waxes by TLC. The chromatographic patterns of reference and unknown mate-

rials are studied. The number, location, and color of spots are noted. The identification is based on how closely the pattern of the unknown sample matches the pattern of a reference material.

     The wax samples are dissolved in chloroform, then spotted on an HPTLC silica gel plate. The plate is developed in a sandwich chamber containing a petroleum ether, diethyl ether, and acetic acid solvent system. The separated components of each wax sample are visualized by spraying with an anisaldehyde detection reagent. The plate is heated after spraying, then examined under ultraviolet light, where some components of the waxes are seen as fluorescent spots.

## Sample Preparation

Reference and unknown sample solutions are prepared in a concentration of 10 μg/μl. The reference sample solutions are made by dissolving 800 μg of a reference material in 80 μl of chloroform. The reference materials include the following waxes: candelilla, carnauba, ceresine, earth, Japan, montan, paraffin, rice, spermaceti, bleached beeswax, white beeswax, and yellow beeswax.

     All solutions are stored in 250-μl insert vials, inside Teflon capped vials, optimal for preventing evaporation of the solutions. The samples are heated at 40 °C for 30 minutes before they are applied to the chromatographic plate.

## Technique

All samples are prepared in reagent grade chloroform. The samples are analyzed on 10 × 10 cm HPTLC silica gel plates (Merck silica gel 60 $F_{254}$). The plates are washed with reagent grade methanol and acti-vated for 1 hour in a 100 °C oven, and are used within 24 hours after activation. Samples are spotted using glass micropipettes, on the baseline, 1 cm above the bottom of the plate.

     For wax analysis, the solvent system for the development is a petroleum ether : diethyl ether : acetic acid (90:10:1) mixture. The development of the chromatographic plate is done in a sandwich chamber. Five ml of the solvent system is placed in the trough of the chamber. The chromatographic plate is clamped to the backing plate and placed in the trough. The whole assembly is placed inside a cylin-drical chamber that is covered to prevent the solvent front from being affected by air drafts. The plate is developed for 8 cm, which usually takes 10–15 minutes.

     The plate is removed from the chamber and allowed to dry in the hood at room temperature for 30 minutes. The separated components of the wax samples are visualized by spraying the plate with an **anisaldehyde-sulfuric acid detection reagent.**



Anisaldehyde

The detection reagent is made by combining 0.5 ml of anisaldehyde, 10 ml of acetic acid, 84.5 ml of methanol, and 5 ml of concentrated sulfuric acid. The reagent remains stable for several weeks when stored in a laboratory refrigerator. Anisaldehyde-sulfuric acid is a universal detection reagent for natural products and makes color differentiation in these materials possible. The background acquires a reddish coloration if the plate is heated too long. It can be decolorized again by interaction with water vapor (Jork et al. 1990). After the plate is sprayed with the detection reagent, it is dried again in the hood for 15–30 minutes, then placed in the oven and heated between 90 and 125 °C for 1–15 minutes. Separation zones of various colors are seen on an almost colorless background and are often fluorescent under long-wavelength UV light.

## Applications

Wax samples from both facsimile and actual museum objects have been analyzed by TLC. One sample for wax analysis came from an encaustic facsimile painting (shown in Figure 6.1) made for testing the analytical techniques under development. The sample was taken from the edge of the panel. A sample solution of 16 µg/µl was made by dissolving 810 µg of the sample in 50 µl of chloroform.

A second unknown sample was taken from a lining adhesive on *Water Lilies* by Claude Monet, painted circa 1920 (Museum of Modern Art, New York), shown in Figure 6.2. When the painting was brought into the conservation laboratory for technical examination, a sample was taken. The lining adhesive was thought to be a wax-resin mixture. The unknown sample solution, whose concentration was 26 µg/µl, was made by dissolving 1.3 mg of the sample in 50 µl of chloroform.

Wax binders were identified by comparison to known materials. Unknown samples included material from the encaustic facsimile painting, and relining material from the Monet *Water Lilies*. Twelve reference waxes were analyzed on the same chromatographic plate with the unknown samples. Figure 6.3 shows the thin-layer chromatogram for the identification of wax binders.

Identification of the individual components of each wax was not attempted. Instead, the chromatographic patterns of the unknown samples were compared to the reference waxes. Separation of at least nine spots was seen for bleached beeswax, white beeswax, and yellow beeswax, spotted in lanes 11, 12, and 13, respectively. This distinct pattern is also seen in lanes 9 and 10, which contain the encaustic facsimile and Monet painting samples, respectively. No other reference material displays this pattern, although each has a characteristic pattern of its own.

The encaustic facsimile painting was manufactured for the Scientific Program at the Getty Conservation Institute as a study piece for



**Figure 6.1.**

A photograph of the encaustic facsimile prepared for binding media analysis. A sample was taken from the edge of the facsimile for wax analysis by TLC.

**Figure 6.2.**

A photograph showing *Water Lilies*, ca. 1920, by Claude Monet (collection of the Museum of Modern Art, New York). A sample of lining material was taken from the painting for wax analysis by TLC.

the validation of analytical methods. The binder used in the portrait was beeswax, as confirmed by this analysis. Also, the relining material of the Monet *Water Lilies* was identified as beeswax. This result was confirmed by gas chromatography/mass spectrometry.



**Figure 6.3.**

TLC analysis of reference waxes, a sample taken from the encaustic facsimile, and an unknown sample of relining material taken from Monet's *Water Lilies*. Stationary phase: Merck HPTLC silica gel 60 $F_{254}$. Mobile phase: petroleum ether : diethyl ether : acetic acid (90:10:1). Detection reagent: anisaldehyde in acidifed ethanol. Lanes: (1) candelilla wax, (2) carnauba wax, (3) ceresine wax, (4) earth wax, (5) Japan wax, (6) montan wax, (7) rice wax, (8) synthetic spermaceti wax, (9) encaustic facsimile, (10) Monet painting, (11) bleached beeswax, (12) white beeswax, (13) yellow beeswax, and (14) paraffin wax.
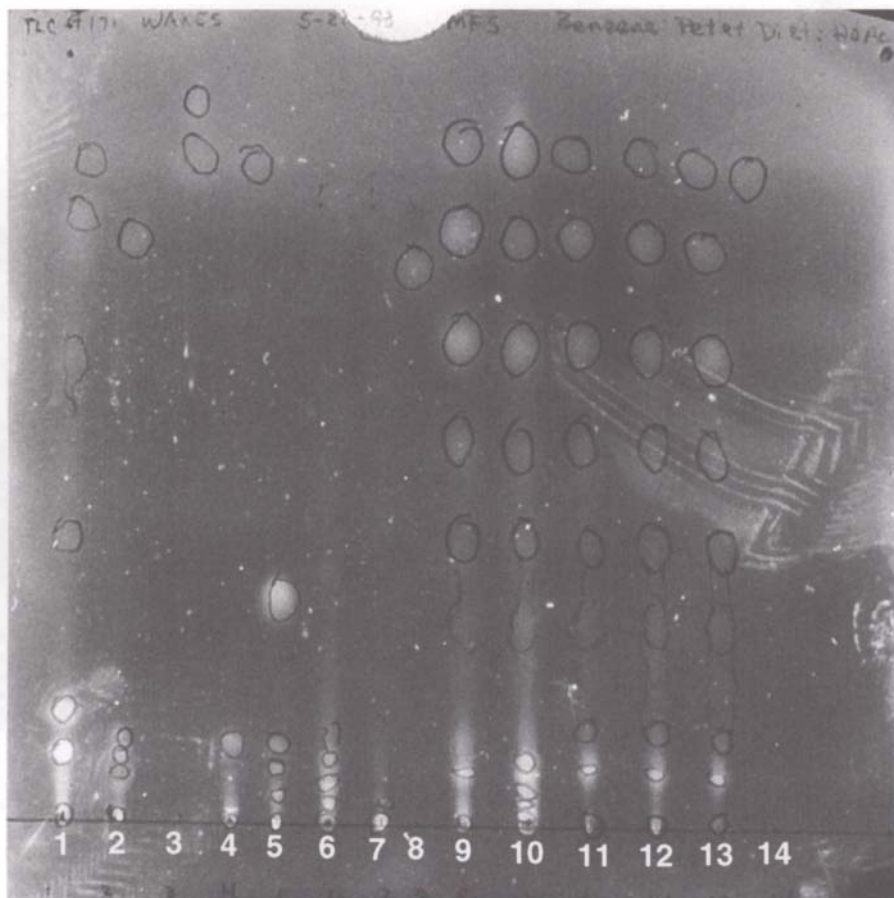
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# Chapter 7

## Analysis of Resins by Thin-Layer Chromatography

The purpose of this chapter is to present:

- A summary of the history and chemistry of resins as it pertains to artists' materials
- A description of the methodology, sample preparation, and technique for using TLC to analyze resin coatings
- Examples of the application of the technique to resin samples from an actual artifact

### A Summary of the Use and Chemistry of Resin Coatings

A detailed presentation of the chemistry and analysis of natural resins and lacquers is given in chapter 8 of *The Organic Chemistry of Museum Objects* (Mills and White 1986).

Natural resins are most often used as varnishes for paintings or furniture, or as a component of a complex binding medium. The resin coating provides an increase in the gloss and translucence of a paint or surface. Mastic from the Greek islands and dammar from Southeast Asia are high-quality natural resins used as picture varnishes. Also, paint media can be made from a mixture of oil, mastic, and wax. Other resins, such as dragon's blood or orange shellac, are used as a colored varnish for musical instruments, or applied as a glaze on gold or silver leaf. Shellac, made from the secretions of lac insects, is used for furniture finishing and for primers on the grounds of paintings (Kuhn 1986).

Some natural resins are water-insoluble by-products exuded from plants and trees, while others are natural lacquers that are water-oil emulsions tapped from trees. Most resins are classified as **terpenoids**, compounds made up of isoprene units (Mills and White 1986). Natural resins contain mono-, sesqui-, di-, and triterpenoids. **Monoterpenoids** are found in oil of turpentine, although the composition depends on the source of pine resin used. Other oils, such as camphor or the oil of rosemary, are monoterpenoid products. **Diterpenoid** resins are found in a larger group of materials, including Canada balsam, rosin, colophony, sandarac, copals, and copaibas. Dammars and mastic are examples of **triterpenoid** resins. Elemis contain a good deal of liquid **sesquiterpenes**, as well as monoterpenes.

## Analytical Methodology

Of the types of binding media studied in this research, resins are the most difficult to identify and differentiate by thin-layer chromatography because there are more components per resin than for other binding media, and it is problematic to obtain complete separations. The resin solutions are prepared by dissolving samples in ethyl acetate. The analysis is performed on silica gel plates in a sandwich chamber, using a benzene and methanol solvent system. The separation of components with the resin samples improves with multiple developments. The chromatographic plates are usually developed two or three times in the same solvent system to improve the resolution of the spots. The plate is sprayed with an antimony trichloride detection reagent and examined under ultraviolet light to visualize the pattern of fluorescent spots for each sample.

## Sample Preparation

Sample preparation involves the dissolution of the unknown resin sample or the reference resin in an appropriate solvent. Ethyl acetate was found to effectively dissolve the resins of interest (Gettens and Stout 1966). The reference resins in these studies included the following: amber, benzoin, colophony, Congo copal, dammar, dragon's blood, elemi, gamboge, Manila copal, mastic, myrrh, sandarac, and shellac. All reference resin samples are prepared in a concentration of approximately 3 mg per ml by adding 6 mg of the material to 2 ml of reagent grade ethyl acetate. The soluble portion of the resin goes into solution in ethyl acetate, while the insoluble portion settles to the bottom of the vial. All unknown sample solutions are made at a concentration of 5 μg per μl in reagent grade ethyl acetate. Both the known and unknown resin solutions are prepared at least 24 hours before analysis.

## Technique

HPTLC silica gel plates (Merck, $10 \times 10$ cm silica gel 60 $F_{254}$) are used to analyze resins. The plates are prepared, cleaned, and activated, following the same procedure used for carbohydrates and waxes. Sample solutions are spotted on the origin line, using glass micropipettes.

Resin analysis uses a benzene : methanol (95:5) solvent system in a sandwich chamber and with a multiple development technique. The chromatographic plate is developed once to a distance of 8 cm, which usually takes 30 minutes. Then it is removed and dried for 30 minutes in the hood. The plate is placed in the same sandwich chamber a second time, and the solvent front travels up the plate again (in the same direction) for the same distance. The plate may be dried and developed a third time.

The chromatographic plate is sprayed with an antimony trichloride detection reagent. It can be purchased commercially in solution, ready to use as a detection reagent for TLC analysis. Or the solution can be prepared in the laboratory by dissolving 10 g of antimony (III) chloride in 50 ml of chloroform or carbon tetrachloride. The solution is sprayed evenly over the TLC plate. The plate is dried, heated at 110–120 °C for 5–10 minutes, and examined under ultraviolet light. Antimony trichloride forms colored $\pi$-complexes with double bond systems.
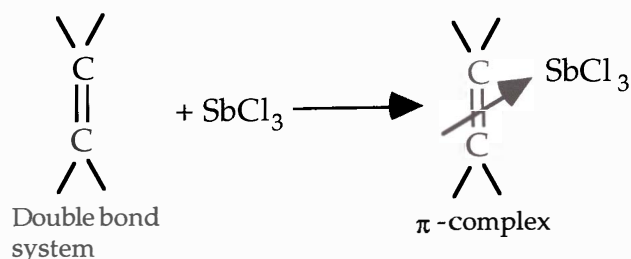


Under long-wavelength UV light, variously colored fluorescent separation zones are seen. Antimony trichloride is the preferred detection reagent for natural resins and balsams, but similar results can be obtained with antimony pentachloride. **NOTE: Both detection reagents are corrosive and toxic and should be handled with extreme care.**

## Applications

Four unknown resin samples were analyzed. Another sample was taken from the Monet relining mentioned in Chapter 6, and prepared in the ethyl acetate carrier solvent. The second sample was an adhesive from Pietre Dure's *Portrait of Pope Clement VII,* shown in Figure 7.1. The third was a varnish sample from a late nineteenth-century urn from the Mathews' workshop (Oakland Museum), shown in Figure 7.2. The fourth sample, seen in Figure 7.3, was from the finish on a German rolltop desk, circa 1785, attributed to David Roentgen. All unknown sample solutions were made at a concentration of 10 μg per μl in reagent grade ethyl acetate.

Resin analysis by TLC is often difficult to interpret, and the following examples highlight the care with which the chromatograms must be evaluated. Two resin analyses by thin-layer chromatography are shown. Figure 7.4 shows the thin-layer chromatogram for the analysis of the resin component of the Monet relining material. Figure 7.5 shows the thin-layer chromatogram for the analysis of the finishes of the Mathews' urn and the Roentgen desk, as well as the adhesive used on the Dure portrait. These chromatograms contain a large number of spots that fluoresce in a variety of colors. Both the locations of the spots and the colors are significant. Identification is based on comparison of the unknown to the reference materials.

**Figure 7.1.**

A front and back view of the *Portrait of Pope Clement VII* by Pietre Dure (J. Paul Getty Museum, 92.SE.67). A sample was taken from the adhesive of the portrait for resin analysis by TLC.



**Figure 7.2.**

A photograph of two nineteenth-century urns from the Mathews' workshop (collection of the Oakland Museum). A sample was taken from the finish of one urn for resin analysis by TLC.

**Figure 7.3.**

A photograph of German rolltop desk, ca. 1785, attributed to David Roentgen (J. Paul Getty Museum, 72.DA.47). A sample of the finish was taken from an interior edge of a drawer of the desk for resin analysis by TLC.



**Figure 7.4.**

TLC analysis of reference resins and an unknown sample of relining material taken from Monet's *Water Lilies* (spotted at several volumes). Stationary phase: Merck HPTLC silica gel 60 $F_{254}$. Mobile phase: benzene : methanol (95:5). The plate was developed three times before detection. Detection reagent: Sigma Chemical antimony trichloride reagent. Lanes: (1) amber, (2) benzoin, (3) colophony, (4) Monet painting, (5) Congo copal, (6) dammer, (7) dragon's blood, (8) Monet painting, (9) elemi, (10) gamboge, (11) Manila copal, (12) Monet painting, (13) mastic, (14) myrrh, (15) sandarac, (16) shellac, (17) bleached beeswax, and (18) Monet painting.
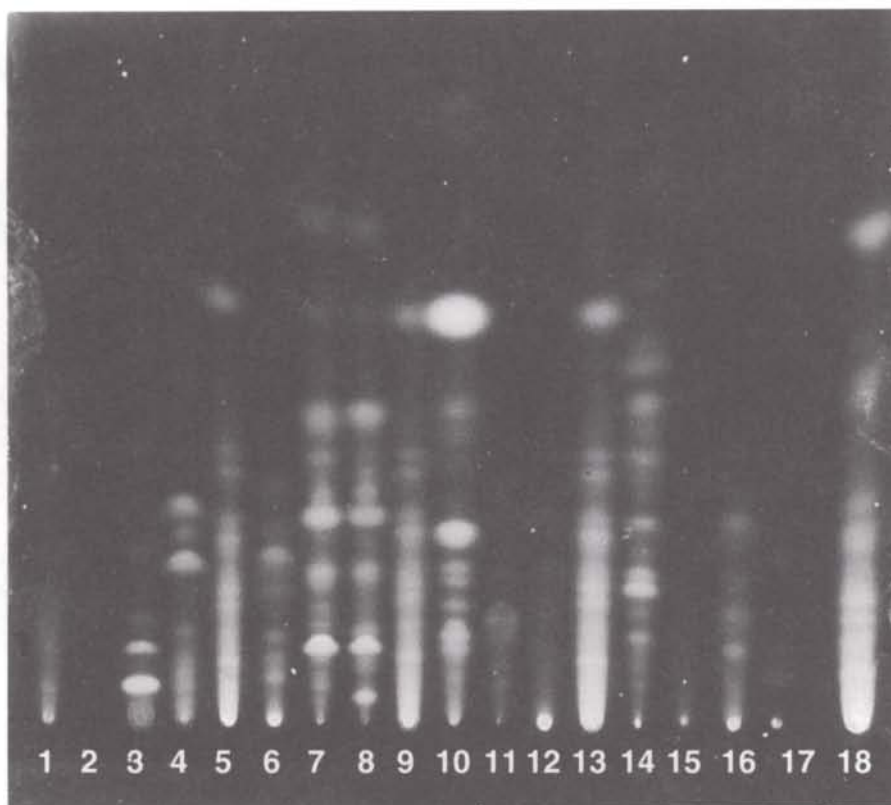
**Figure 7.5.**

TLC analysis of additional resin samples. Stationary phase: Merck HPTLC silica gel 60 $F_{254}$. Mobile phase: benzene : methanol (95:5). The plate was developed three times before detection. Detection reagent: Sigma Chemical antimony trichloride reagent. Lanes: (1) amber, (2) benzoin, (3) colophony, (4) Congo copal, (5) dammer, (6) dragon's blood, (7) elemi, (8) gamboge, (9) Manila copal, (10) mastic, (11) myrrh, (12) shellac, (13) sandarac, (14), Pope Clement adhesive, (15) Mathews' urn finish, and (16) Roentgen desk finish.
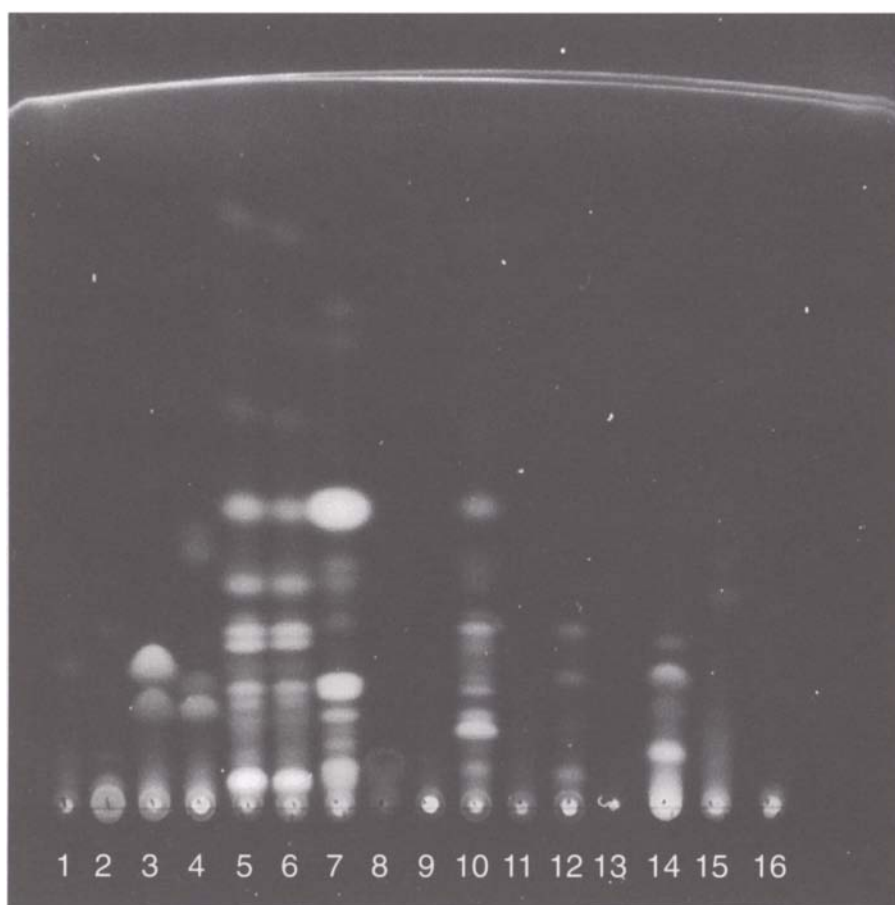
The chromatograms were developed three times. Threefold development improves the separation of the components within each sample, but the solvent front shows more curvature than in a twofold development. Depending on the nature and the need for resolution within the sample, two- or threefold development can be used. In many situations, an identification of the resin can be made after two developments.

The Monet sample, analyzed in the chromatogram shown in Figure 7.4, was spotted at four different volumes. Lanes 4, 8, 12, and 18 of the chromatogram contain 0.2, 0.5, 1.0, and 2.0 µl of the sample, respectively. The chromatographic pattern of this sample shows fourteen or more spots, most of which have $R_f$ values less than 0.422. This pattern most closely resembles that of elemi, located in lane 9 of Figure 7.4. Most obvious is the intense yellow spot located in each lane with an $R_f$ of 0.518. The differences between the Monet sample and the reference elemi are probably due to the presence of other resinous additives within the Monet sample. Several spots in lane 12 of the Monet sample's chromatogram are similar to the mastic sample in lane 13. Both mastic and gum elemi are known to be components of the "Dutch process" wax-resin relining mixtures (Plenderleith and Cursiter 1934). The presence of mastic in the Monet sample was confirmed by both FT-IR microscopy and gas chromatography/mass spectroscopy.

The *Pope Clement* sample in lane 14 of Figure 7.5 contains some spots similar to that of the colophony resin (a balsam) in lane 3. An intense blue spot seen in the sample is not analogous to any material on the plate. It may be due to an impurity or an additive in the resin. Alternatively, the reference material may not be a good match because there are multiple types of balsams or pine tree resins that can vary in their composition due to the source and type of tree. While the analysis of the sample by FT-IR microscopy confirms that the material is a balsam, there is also the possibility that the blue spot is due to an added component that was below the detection limit of the IR.

The finish on the Mathews' urn cannot be identified by comparison to the reference materials on this plate. The solubility of the material and the complex pattern of fluorescent spots seen in the chromatogram display characteristics of a resinous material, but the sample does not match any reference. Information supplied later by the conservator of the piece indicated that the resin may be bitumen. Further analysis with bitumen or other reference materials may identify the finish.

The chromatogram for the Roentgen desk finish sample in lane 16 resembles the pattern seen in lane 12 for shellac. It shows two spots that are distinctive of a shellac located near the origin of the plate. The first, at the origin, has a distinctive orange color associated with a shellac resin. Other spots seen in the Roentgen desk sample may be due to the presence of waxes or other impurities, since waxes can also be detected with the antimony trichloride detection reagent. The presence of shellac was confirmed by FT-IR microscopy.

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# Chapter 8

## Visualization, Interpretation, Documentation, and Computer Analysis of Chromatographic Plates

The purpose of this chapter is to present:

- An overview of visualization reagents for use in detection of TLC spots
- A discussion of qualitative and quantitative methods of interpretation of TLC plates
- A description of proper techniques for documentation of TLC experiments
- Information on the application of computer imaging for the evaluation of the TLC plate

## Visualization Reagents

Once the components of a sample are separated, we must be able to recognize where the spots for each component lie on the plate. Some components may be colored compounds and easily recognized, while others may be colorless and must be detected by other means. Visualization, which is based on physical or chemical principles, may be destructive or nondestructive to the separated molecules. An nondestructive method of visualization leaves the component unchanged after detection, while a destructive method permanently alters the component.

### Physical Methods

These visualization methods are based on plate observation under ultraviolet radiation. This can be used in two ways. One type of spot location involves the absorption of UV radiation, and the subsequent fluorescence, by the component. If the TLC plate contains no fluorescent reagent, the component will be seen as a bright spot on a white background, meaning that the component fluoresces under these conditions. A second way of locating the component is carried out by adding a fluorescent reagent to the TLC plate. With this method, nonfluorescing components are seen as dark spots on a fluorescing background.

In both physical methods, a UV lamp is needed that is capable of emitting radiation at short wavelengths (264 nm) and long wavelengths (366 nm). Mercury vapor lamps are usually used as the UV

source. Less intense fluorescent tube lamps can also be used. Several laboratory UV lamps are specially designed for the examination of TLC plates. They range from handheld models to those in elaborate cabinets designed for viewing and photodocumentation.

## Chemical Methods

These visualization methods involve the reaction of a detection reagent with the sample components; detectable substances (derivatives) are formed. The chemical detection reagent reacts with the compound, rendering it either visible under normal light or fluorescent under UV light. The chemical detection reagent can be reacted with the compound during sample preparation, at the start of the chromatographic separation, or after chromatographic development. The third method, called postchromatographic detection or derivatization, is the most common way of visualizing separation zones.

The aims of postchromatographic derivatization include:

- facilitating the detection of colorless separation zones;
- increasing the selectivity of the detection; and
- improving the detection sensitivity.

The detection reagent may be a vapor or a liquid. Liquid detection reagents may be sprayed onto the plate, or the plate may be dipped into the reagent. The reaction between the detection reagent and the chromatogram may result in a permanent change to the separated components, or it may be transient in nature and leave the separated component unchanged. These chemical detection reagents can be universal reagents or substances that react with particular functional groups.

**Spraying** is the most common method of applying the detection reagent to the TLC plate. A glass sprayer is normally used and is connected to a pressure supply, such as a hand pump or a gas cylinder. Spraying can be carried out at pressures of 0.6–0.8 bar, from a distance of 20–30 cm in a suitable spray booth or fume hood. The spray is applied in the "back and forth, up and down" pattern shown in Figure 8.1. After the plate is sprayed, the glass sprayer and the spray booth should be carefully cleaned so that undesired reactions do not occur between detection reagents. **Some detection reagents should**
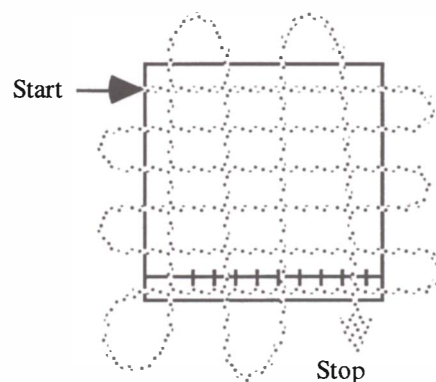


**Figure 8.1.**

Spray pattern for the application of the visualization reagent.

never be sprayed because they can cause explosions in the exhaust duct of the fume hood or the spray chamber. These detection reagents include manganese heptoxide- and perchloric acid-containing reagents, sodium azide, and iodine azide solutions.

**Dipping** is a viable alternative to spraying as a method of application. Dipping usually results in a more homogeneous coating than careful spraying. It also provides an increased reproducibility in the detection of the separated compounds. Dipping can be performed in a conventional TLC developing chamber, but the required volume of detection reagent for dipping is usually prohibitive. Low-volume dipping chambers and automated, time-controlled dipping apparatus are commercially available. Care should be taken in the choice of solvent used in preparing the detection reagent, since some separated substances may dissolve and subsequently be removed from the TLC plate before they can be visualized. The dipping time should be 5 seconds or less.

A third method of application is exposure of the TLC plate to the vapors of the detection reagent. This is commonly done with a detection reagent such as iodine. Iodine vapors usually stain organic compounds, making spots yellow and leaving the background white. Iodine vapors are a nonspecific, usually nondestructive way of detecting many substances.

Almost all chemical reactions proceed more rapidly at elevated temperatures. TLC plates treated with a detection reagent should usually be heated to promote the desired reaction. Heating to 100–120 °C for 5–10 minutes is usually sufficient to complete the reaction. The plate should be heated as evenly as possible to ensure a homogeneous reaction. Heating of the plate can be performed with care in laboratory ovens or on hot plates. The advantage of the hot plate is that it allows for the direct viewing of the reaction. **NOTE: The hot plate must be placed in a fume hood away from flammable solvents to prevent exposure to hazardous fumes that may be emitted from the surface of the TLC plate.**

Specific detection reagents that are used for analysis of artists' materials have been described in previous chapters. We selected them for their sensitivity of detection, and in some cases for their relatively low toxicity. For example, the "best" detection reagent for monosaccharides is a solution of aniline and phthalic acid. Aniline is a known carcinogen and is difficult to handle carefully. Aminohippuric acid was chosen instead because it provides good detection of monosaccharides and is a much less hazardous material. In other cases, as with the detection of resins, no viable alternative was found to antimony trichloride, which requires extreme care in handling. Other detection reagents have been reported in the literature for the detection of amino acids, sugars, terpenes, and other components similar to those found in binding media. Several literature sources (Bruno et al. 1989; Jork et al. 1990; Touchstone and Dobbins 1983a) provide detailed information on the use and preparation of detection reagents.

## Interpretation of the TLC Plate

The interpretation of a TLC plate can be a qualitative or a quantitative process, depending on the particular analysis. For example, the individual components of a hydrolyzed carbohydrate sample can be quantified using standard solutions of monosaccharides. But quantification is not necessary in order to distinguish between most carbohydrate binders, coatings, or adhesives. The quantification of individual amino acids in a protein sample, alternatively, may provide information that cannot be elucidated simply by noting the presence or absence of certain amino acids. The following sections describe qualitative and quantitative methods of evaluating a TLC plate.

### Qualitative Methods

A major application of TLC is the qualitative identification of compounds, based on the numbers, colors, and $R_f$ values of separation zones (Sherma 1991). Identification can be aided by using more than one detection reagent, often applied in sequence to a single chromatogram. For example, the visualization of proline and hydroxyproline with ninhydrin results in yellow spots that can be difficult to notice. If the plate is sprayed with an isatin detection reagent after detection with ninhydrin, the proline and hydroxyproline separation zones can be seen as blue spots on a yellow background.

$R_f$ values of the separation zones are compared to those of reference materials for identification of unknown samples. The $R_f$ value is easily determined with an accurate ruler. The first step in calculating the $R_f$ value is the measurement of the location of each separation zone. This value is measured from the origin of the plate to the center of the separation zone, $d_1$, as shown in Figure 8.2. Next, the distance from the origin to the solvent front, $d_s$, is measured. The $R_f$ value is calculated by dividing the distance to the center of the separation zone by the distance to the solvent front, $d_1/d_s$.

An example of this calculation is shown in Figure 8.3. Another method for the determination of $d_1$ is to measure the distance from the origin to the nearest edge of the spot, $s_1$, and the distance from the origin to the farthest edge of the spot, $s_2$; then average the two values to find the center distance. An example of this calculation is shown in Figure 8.4.

Once the $R_f$ values of the components of the sample and reference materials are determined, they must be compared. One way to do this is to prepare a table listing the $R_f$ values for each sample or reference material in one column and the corresponding color of the separation zone in a second column. An example of this method is shown in Figure 8.5. The pattern of $R_f$ values and colors for the unknown sample may match one of the reference materials. The $R_f$ values should agree within ±3% compared to the reference material chromatographed under the same conditions (Sherma 1991). If the chromatogram does not match any reference material, it may be reanalyzed using different reference materials.
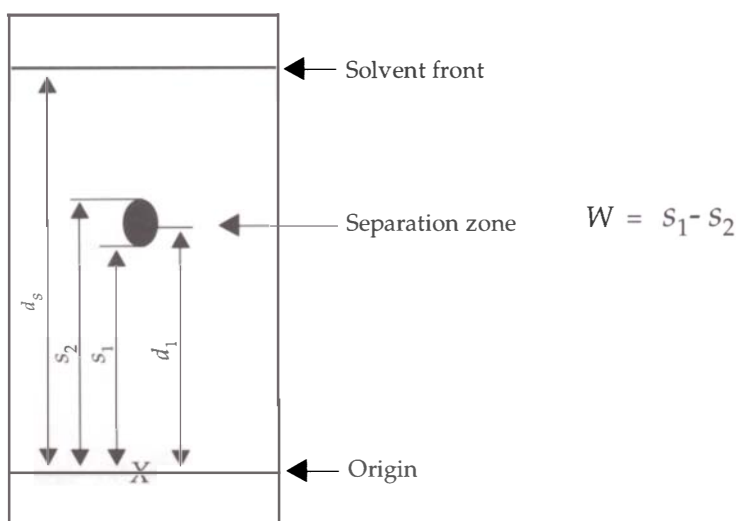
**Figure 8.2.**

An illustration of a TLC separation zone, indicating the origin, separation zone, and solvent front.

$$W = S_1 - S_2$$

For confirmation of an identification by TLC, the unknown sample and reference materials can be separated using a TLC system with a different separation mechanism (e.g., adsorption, partition, reversed phase). The $R_f$ values of unknown and reference materials are compared for the second separation and should result in the same identification.

## Quantitative Methods

There are several methods for the quantification of a solute by TLC, including visual estimation, zone elution, and scanning densitometry.

The simplest method for semiquantitative analysis is a visual evaluation of the size and intensity of the spots of the unknown sample compared to known standard solutions. A series of standard solutions is made by accurately weighing samples of reference materials similar to components thought to be in the sample. These solutions are spotted on the same plate as the unknown sample and chromatographed under identical conditions. A template is used to estimate the size of the separation zones for unknown and standard samples. A
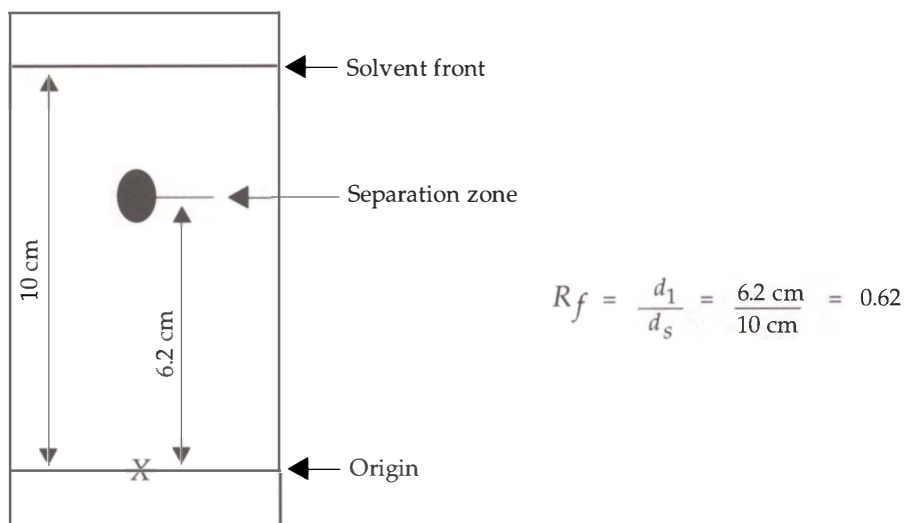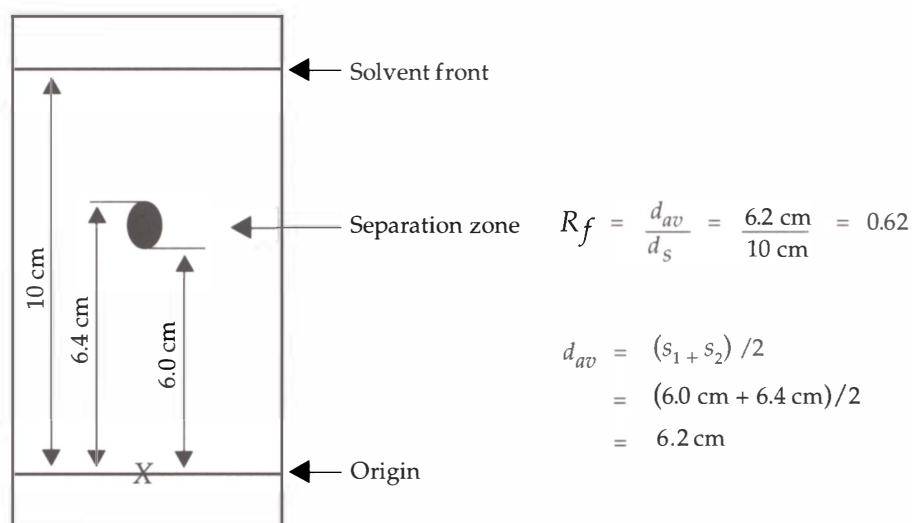


**Figure 8.3.**

Calculation of the $R_f$ value by the center spot method.

$$R_f = \frac{d_1}{d_s} = \frac{6.2 \text{ cm}}{10 \text{ cm}} = 0.62$$

**Figure 8.4.**

Calculation of the $R_f$ value by the averaging method.

$$R_f = \frac{d_{av}}{d_s} = \frac{6.2\ cm}{10\ cm} = 0.62$$

$$d_{av} = (s_1 + s_2)\,/2$$
$$= (6.0\ cm + 6.4\ cm)/2$$
$$= 6.2\ cm$$

clear template has a series of circles of varying diameters marked on it. The template is positioned over the separation zone so that the diameter of the circle is matched to the diameter of the spot. Once the diameter of each spot is recorded, the intensity of the spot is visually ranked. By comparing the size and intensity of the spots found for the unknown sample to spots of standard reference solutions, a relative amount of the components of the unknown sample can be estimated.

For this technique to succeed, good separation of zones is needed, and the $R_f$ values of the zones must be large enough so that the spot is not too concentrated. Solutes with $R_f$ values between 0.3 and 0.7 are readily evaluated by this method. The visual comparison method works best if amounts near the detection limit are applied and if the sample is closely bracketed with standards. This method has the accuracy and reproducibility in the 10–30% range, and has seen little use in recent years due to the appearance of more accurate methods, such as scanning densitometry. The advantage of the visual estimation method is that little instrumentation is needed. As stated, disadvantages include lower accuracy and poor reproducibility.

**Zone elution** is another solute quantitation method and is described in detail elsewhere (Sherma 1991; Touchstone and Dobbins 1983c). A brief summary of the method is presented here. The zone elution method involves the removal of the individual separation zones from the TLC plate and quantification by an independent microanalytical method, such as solution spectrophotometry, gas chromatography, or

**Figure 8.5.**

An example of a data sheet for documenting TLC data.

| Spot | Unknown 1 | | Reference 1 | | Reference 2 | |
|------|-----------|-------|-------------|-----------|-------------|-----------|
|      | $R_f$     | Color | $R_f$       | Color     | $R_f$       | Color     |
| 1    | 0.42      | blue  | 0.38        | orange    | 0.42        | blue      |
| 2    | 0.51      | blue-green | 0.52   | blue-green | 0.51       | blue-green |
| 3    | 0.79      | pink  | 0.81        | purple    | 0.79        | pink      |

### Optical Methods

Optical methods of quantitative analysis fall into two categories—those based on measuring the change in transmittance or reflectance of the chromatogram caused by the presence of the component; and those based on the transformation of the illuminating light energy into longer wavelength energy, such as fluorescence by the component.

Instruments for scanning densitometry are basically optical systems that have a light source, condensing and focusing systems, and a photosensing detector. They are usually capable of adsorbance, reflectance, or fluorescence measurements in a spectral range from 200 to 800 nm. The instruments use two light sources to cover this spectral range, a deuterium lamp for the UV region, and a tungsten or tungsten-halogen lamp for the visible region. In addition, a mercury vapor lamp is used to provide sufficiently high energy for scanning by fluorescence.

The following will describe the process of densitometry from the illumination of the plate through the detection of the signal to the calculation of the concentration of the components.

Densitometers can be operated in a transmission or a reflection mode. In the transmission mode, the TLC plate is illuminated from the glass side of the plate, and the photosensitive detector is placed on the sample side of the plate. The light passes through the plate before it is detected. In this arrangement, the presence of a separation zone causes a change in the intensity of the light source. In the reflection mode, the light source and the detector are located on the same side of the TLC plate. In this case, the light source is reflected from the surface of the TLC plate. The presence of a separation zone alters the amount of light reflected. In general, transmission

voltametry. In the first step of the zone elution method, the layer is fully dried to remove any mobile phase. Next, the separation zones are removed by scraping and collecting the layer containing the solute. Then the solute is eluted from the sorbent layer with appropriate solvents. Finally, the concentration of the solute in the solution is determined. Zone elution is a tedious and time-consuming method, and is likely to be inaccurate due to loss of sorbent during scraping and collection, background interferences, or difficulties of locating exact zone boundaries.

**In situ scanning densitometry** is the most commonly used method of quantitation and involves plotting the adsorbance or fluorescence of light from a scanned lane of the TLC plate. The plate is scanned with a beam of light and a detector. The intensity of the light reaching the detector changes in the presence of a separation zone. This change is a function of the amount of analyte present in the separation zone. The amount of any given component in an unknown sample is determined by comparing a plot of its separation zone to a standard calibration curve made from plots of reference materials chromatographed on the same plate.

## Documentation

The information produced in a TLC separation can be recorded for storage or documentation as a manual-graphical reproduction, a photocopy, a photograph, or an autoradiograph. Also, the plate can be scanned using computer techniques, and the raw data can be stored in databases. This section describes general and photographic methods of documenting a TLC plate. Written records of the conditions under which a chromatogram is made are also discussed.

### Manual-Graphical Methods

The simplest form of documentation is the preparation of a list of $R_f$ values for each sample. Often, listing the $R_f$ values does not give a true picture of the chromatogram because there is no description of the size and shape of the separation zones. The list can be augmented with a description of the color of the zones.

A tracing of the chromatogram can be made, and the shapes of the separation can be carefully hand drawn. Colors can be denoted using a hatch-mark system (Touchstone and Dobbins 1983b). This denotation can also be used on photocopies of the chromatogram. Either the tracings or photocopies can be adhered into a research notebook using archival tape or glue. This method is a good choice when the color of the spots is subject to fading over time.

### Photographic Methods

An excellent description of photography for documentation of TLC plates is provided elsewhere (Vitek 1991). Photography is the preferred method for permanently documenting a chromatogram. Standard

modes are used if the layer medium is clear, as in gel electrophoresis. With most TLC plates the sorbent layer is too turbid to permit transmission measurements. The reflection mode becomes the method of choice.

The separation zones are spatially distributed over the surface of the TLC plate. This spatial information is turned into a time-dependent signal by scanning the plate with the light beam in the form of a slit or a point. One-dimensional separations are usually scanned lane by lane, where the lane is the rectangular area defined by the length of the development and the width of the spots. The lane can be scanned in a variety of patterns from a zigzag to a meandering scan.

The diffusely reflected light is measured by the photosensor of the densitometer. This photosensor may be a photomultiplier tube, a solid state diode, a vidicon detector, or a self-scanning charge coupled device (CCD) camera. The difference between the intensity of the light coming from the sample zone and the intensity of the light from the sample-free background on the plate is measured. This value is then compared with values for calibration standards located on the sample plate. The conversion of the analogue signal to digital signal, the integration of the signal, and the comparison of signals are performed by the computer. The way in which each step is performed adds a margin of error to the final result. These potential errors are taken into consideration in the calculations. Additional details on the theoretical aspects of densitometry can be found in the literature (Pollak 1991).

black-and-white, color, and instant-image photography can be used to record the chromatogram under normal or ultraviolet illumination.

A basic 35-mm photographic system for documenting a TLC plate includes a 35-mm camera with a standard and a macro lens, a series of filters, a copy stand, a light box, two tungsten lamps, two ultraviolet lamps, and a "UV box." Figure 8.6 illustrates a typical setup for documentation of a chromatogram using a 35-mm camera. Recommended films for documentation include Kodak 160 ASA tungsten-balanced slide film, Kodak TMAX 100 and 400 ASA black-and-white films, and Fujichrome 400 ASA daylight-balanced slide film. Other films may be used, but keep in mind that color correction filters may be needed to obtain accurate color.

Chromatograms that display visible separation zones are usually documented under normal light with Kodak 160 ASA tungsten-balanced slide film and Kodak TMAX 100 ASA black-and-white film. (These slower-speed films are used because of their fine grain and excellent reproduction and enlargement qualities. If light is limited, faster-speed films such as the Scotch 640 tungsten-balanced slide film or the Kodak TMAX 400 ASA black-and-white film may be more effective.) Tungsten lamps are positioned 45° on each side of the plate. A light box (i.e., a slide-viewing box) is placed in the center of the copy stand. The chromatographic plate is placed on the light box. The 35-mm camera, loaded with the appropriate film, is mounted on the copy stand. The exposure is metered with the light meter built into the camera, or with an optional handheld light meter. It is often useful to bracket the exposure by ± 1 f-stop. Slides can be stored in polyethylene sheet holders. Black-and-white negatives can be contact printed and then stored with the print in a polyethylene page protector.

Documentation of a chromatogram, using ultraviolet light (366 nm), is performed with both black-and-white print and color-slide film. The room used for the documentation must be completely sealed from light; a darkroom or an interior room with no windows can be used. Ultraviolet lamps are placed at 45° on each side of the plate inside a UV box. A 35-mm camera with a 50-mm macro lens is used to document the plate. The camera is attached to the arm of the copy stand and is positioned over the opening of the UV box. Other arrangements can be used as may be available. Fujichrome 400 ASA daylight-balanced slide film is shot at 800 ASA and "push-processed." Kodak Wratten gel filters are used to correct color shifts (filters 10R, 10M, and 20Y) and minimize back-reflected ultraviolet radiation (filter 2E). The exposures are based on trial and error. Successful results have been obtained with an exposure range of 4–32 seconds. Kodak TMAX 400 ASA black-and-white film is shot at 800 ASA with a Kodak Wratten 2E filter for black-and-white documentation.

Instant-image photography usually involves the use of Polaroid systems or film. Polaroid has developed a system specifically for the documentation of electrophoresis gels and thin-layer chromatograms. Other systems have been developed that use a Polaroid back attachment that fits on the UV viewer (Vitek 1991).
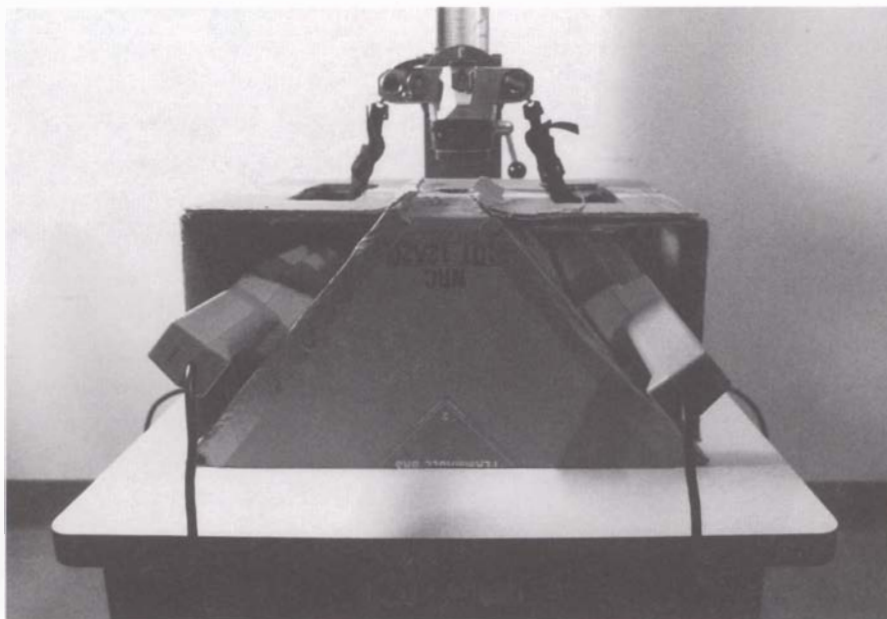
**Figure 8.6.**

Photographic equipment: a simple photographic housing for ultraviolet documentation of thin-layer chromatograms made from cardboard and fitted with two handheld ultraviolet lamps.

## Written Record of Conditions

It is important to keep a written record of the conditions present in any given chromatographic separation. This practice helps facilitate the reproduction of TLC results and procedure publication. A definite effort should be made to report all details of the chromatographic process. Recommended parameters to be included in the documentation are:

- method of development: description of tank, saturation conditions, and method of placing the TLC plate in the tank (i.e., upright versus angled);
- temperature and humidity;
- stationary phase: type of sorbent, brand, batch number, layer thickness, precoated or homemade, type of backing, size of channels, method and temperature of activation, and impregnation;
- mobile phase: complete description of individual solvents and the method of preparing the mixture, volume used, and method of drying after development;
- detection method: method for reagent application, and description of reagent preparation;
- sample solution: weight, extraction, and clean-up procedures, spotting apparatus and technique, sample volume, and derivative preparation; and
- reference solutions (standards): composition, solvent, spotting apparatus and technique, and sample volume.

It is useful to standardize your written documentation by using a form. For further information, see Protocol E.

## Computer Methods for Evaluation of the TLC Plate

This section describes recent research at the Getty Conservation Institute on the use of computer methods of evaluation. Often the identification of binding media by thin-layer chromatography requires the recognition and differentiation of subtle variations in chromatographic patterns. In some cases, identification can be based on the presence or absence of a particular component. In other cases, it is the presence and concentration ratios of particular components that lead to an identification. One way to approach the problem is to quantitatively determine the amount of each component in the sample. Quantitative analysis by thin-layer chromatography can be performed using scanning densitometry, as mentioned above. This method requires expensive equipment and a dedicated computer. Less expensive video densitometers that can be used for quantitative methods are available (Touchstone 1993), but they may still be beyond the budget of the average conservation laboratory.

Part of our recent research involves the development of computer-aided analysis to match chromatographic patterns between known and unknown samples (Striegel, in preparation). This approach assumes that a Macintosh computer is available to the conservator. An image of the chromatographic plate is digitized using a full page or hand scanner, and a density plot of each chromatographic lane of the digital image is determined using NIH Image public domain software on a Macintosh computer. Further analysis results in a series of peak areas associated with the spot locations for each chromatographic lane. The data are analyzed using Statview statistical software. The identification of the unknown binder is made by comparing it to a series of known references to find the pattern that most closely matches.

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# Chapter 9

## Scientific Examination of Works of Art

In previous chapters, examples were given of the application of TLC to the analysis of binders, adhesives, and coatings. These analyses were performed at the Getty Conservation Institute as part of the development of a TLC protocol. Other TLC systems have been used in the analysis of works of art. The purpose of this chapter is to present:

- A selected review of the use of TLC in the analysis of artists' materials
- A description of other TLC systems that may be useful for binding media analysis

### A Review of the Uses of TLC in the Examination of Works of Art

In 1986, L. Masschelein-Kleiner published "Analysis of Paint Media, Varnishes, and Adhesives." This work, which is included in the collected articles section of this book, reviews the use of many analytical techniques including infrared spectroscopy, paper chromatography, thin-layer chromatography, gas chromatography, high-performance liquid chromatography, pyrolysis gas chromatography, mass spectroscopy, and differential thermal analysis. Masschelein-Kleiner's section on thin-layer chromatography presents in tabular form various TLC systems that have been used to identify carbohydrates, proteins, waxes, and natural resins.

### Protein Analysis
The identification of proteinaceous and other binders has been the subject of papers in English (White 1984; Broekman-Bokstijn et al. 1970), Russian (Jelninskaya 1970), Croatian (Antonov 1977), and other languages.

In 1958, one of the earliest works in the analysis of paint media by chromatography was published (Hey 1958). In this paper, Hey describes the identification of proteinaceous binders using paper chromatography. The paint samples were hydrolyzed in a hydrochloric acid : water solution (1:1) at 90–100 °C for 24 hours. The hydrolysate solution was neutralized with an ion exchange column. The samples were spotted on Whatman no. 1 chromatography paper and developed in a mobile phase of n-butanol : glacial acetic acid : water (60:15:25). The separation

zones were visualized with a 0.2% solution of ninhydrin. Isatin was employed as a second detection reagent.

Thin-layer chromatography has also been applied to the identification of proteinaceous and carbohydrate binders in illuminated manuscripts (Flieder 1968). Flieder describes the analysis of seven samples taken from various manuscripts. These samples were hydrolyzed by two methods. The first method, for hydrolysis of proteins, involved the hydrolysis of the samples in a 4% sulfuric acid solution at 100 °C for 20 hours. The sample solutions were neutralized with barium carbonate, and were then separated from the precipitated salts by centrifugation. In the second method, the samples were hydrolyzed in dilute hydrochloric acid (3%, 6%, or 12%) at 105 °C for 2, 15, or 24 hours, depending on the concentration of the acid. The samples were then neutralized by evaporation or by passing the sample solutions through an ion exchange resin column.

The protein hydrolysates were separated on Kodak K 511 polycarbonate plates impregnated with a pH 6.8 buffer. The mobile phase was ethanol : water : ammonia (85:13:2). The separated zones were visualized with a 2% ninhydrin solution in ethanol.

The carbohydrate hydrolysates were separated on Kodak K301V silica gel plates using a propanol : ethyl acetate : water : 25% ammonia (30:5:15:5) mobile phase. The separation zones were detected with 2% naphtoresorcinol in 10% ethanolic phosphoric acid.

In more recent analysis of proteinaceous media, the dansyl derivatives of amino acids were separated on micropolyamide plates (Tomek and Pechova 1992). The derivatives were separated in a water and formic acid (50:1.5) mobile phase, and detected by UV fluorescence at 254 nm wavelength.

## Carbohydrate Analysis

The analysis of carbohydrates by TLC is often mentioned in the papers discussed in the last section. Gums and other binders used in polychrome reliefs in ancient Egyptian limestone tombs have been identified by TLC (Poksinska and Shoeib 1988). In the study, carbohydrates and proteins were identified by paper chromatography, and esters of fatty acids were examined by TLC. The samples for carbohydrate analysis were hydrolyzed with $4N$ sulfuric acid for 6 hours at 105 °C in hermetically sealed test tubes. The hydrolysates were neutralized over barium carbonate and isolated by filtration. The samples were spotted on chromatography paper and developed twice in a mobile phase of ethyl acetate : pyridine : water (3.6:1:1.15). The detection reagent was not identified. The samples were identified as plant gums.

The identification by TLC of plant gums used as binders or adhesives was published in 1989 (Matousova and Bucifalova 1989). Plant-derived reference gums from a variety of geographical locations were obtained as reference standards, including gum arabic, gum tragacanth, and fruit gums. Various methods of hydrolysis and sample derivation are described in the paper. The samples were separated on silica gel layers impregnated with boric acid. A series of mobile phase systems

was tested, including butanol : ethyl acetate : water (60:20:20) and ethyl acetate : pyridine : water (100:35:35). The authors also tested several detection reagents and tabulated the semiquantitative amounts of mono-saccharides in each of the gum hydrolysates.

## Wax Analysis

Fewer applications of TLC have been reported for the analysis of wax samples than for other materials. One previously mentioned article (Broekman-Bokstijn et al. 1970) uses TLC for the identification of beeswax and carnauba wax. These waxes were identified by comparing the spots of hydrocarbons, esters of fatty acids, and the triglycerides found in the unknown samples with those of reference materials.

TLC has been used in the forensic examination of the wax or lac seals (Baggi and Murty 1982) affixed to letters, documents, enve-lopes, parcels, and containers. Twenty-five sealing waxes were collected from various sources. After the samples were extracted into ethanol and gently heated for 15 minutes in a steam bath, they were spotted on silica gel G plates that had been previously activated for 1 hour at 105 °C. The plates were developed to a distance of 10 cm in one of three solvent systems:

1. xylene : butanone : methanol : diethylamine (40:40:6:2)
2. benzene : methanol (95:5)
3. benzene : methanol : acetic acid (90:5:5).

The samples were visualized by examining the plate with UV light at 254 and 366 nm. They were also detected with an anisaldehyde : acetic acid : methanol : sulfuric acid reagent (0.5:10:84.5:5).

## Resin Analysis

An excellent paper on thin-layer chromatography as an aid in the identi-fication of binding materials was presented by Wilma Roelofs in 1972 at the plenary session of the ICOM meeting in Madrid (Roelofs 1972). The paper, titled "Thin-Layer Chromatography: An Aid for the Analysis of Binding Materials and Natural Dyestuffs for Works of Art," describes the general procedures and application of TLC. Roelofs's section on resins describes the analysis of a varnish sample removed from a painting by extraction into chloroform on a cotton swab. The unknown sample was spotted next to reference samples on a silica gel G plate and developed up to three times in chloroform. The separation zones were detected with a 20% solution of antimony pentachloride in carbon tetrachloride. Roelofs also describes chromatographic systems for waxes, gums, and glues. Resin analysis was also described by others (Broekman-Bokstijn et al. 1970).

## Some Potential Systems for Media Analysis by TLC

Most of the analyses discussed in this publication have utilized silica gel plates. As the development of modern methods for TLC progresses, new

modified normal phase and reversed phase plates are becoming available, which may aid in improving the selectivity of chromatographic separations. The following systems represent the most recent developments reported in the technical literature. Due to time constraints in the preparation of this course, these systems have not yet been tested, but they may prove useful in binding media analysis.

Silica gel plates modified with amino groups (Macherey Nagel, Nano-SIL NH$_2$/UV) have been used to separate sugars. One such separation was performed using an ethyl acetate : pyridine : water : glacial acetic acid (60:30:10:5) mobile phase. The sugars that were separated included lactose, saccharose, galactose, glucose, fructose, arabinose, xylose, and ribose. The separation zones were detected with scanning densitometry (Macherey-Nagel 1993).

Reversed phase plates (i.e., RP-18 plates) may be useful in separating sterols and may lead to a method of identification of oils by TLC. The development of this method depends on the quantity of sterols surviving in the dried film, the possible derivatization of the sample, the selectivity of a mobile phase, the sensitivity of a detection reagent, and the semiquantitation or quantitation of the components.

The systems mentioned only scratch the surface of the potential of TLC systems. Modern TLC methods and new instrumental techniques rival the capabilities of HPLC methods.

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Part 2

# Protocols

# Introduction

The following protocols were prepared for the course "Methods in Scientific Examination of Works of Art: Thin-Layer Chromatography." They are a direct result of the Getty Conservation Institute's Binding Media Research Project. These protocols are intended to be step-by-step guides for practical laboratory procedures in the analysis by thin-layer chromatography of artists' materials, such as paint binders, adhesives, and coatings. Each protocol describes the methodology needed for the analysis of proteins, carbohydrates, waxes, or resins.

  The protocols should not be considered the final word on TLC analysis of artists' materials. Other chromatographic systems may yield positive results. As new chromatographic materials and methodologies are developed, the procedures may be modified. Modifications to the protocols may be necessary due to the limitations of equipment or materials. For example, a simple screw-top jar may be used as a developing chamber if the traditional, more expensive TLC chambers are not available. Participants in the course and readers of this publication are encouraged to keep abreast of the research literature and to develop their own research strategies.

Protocol A

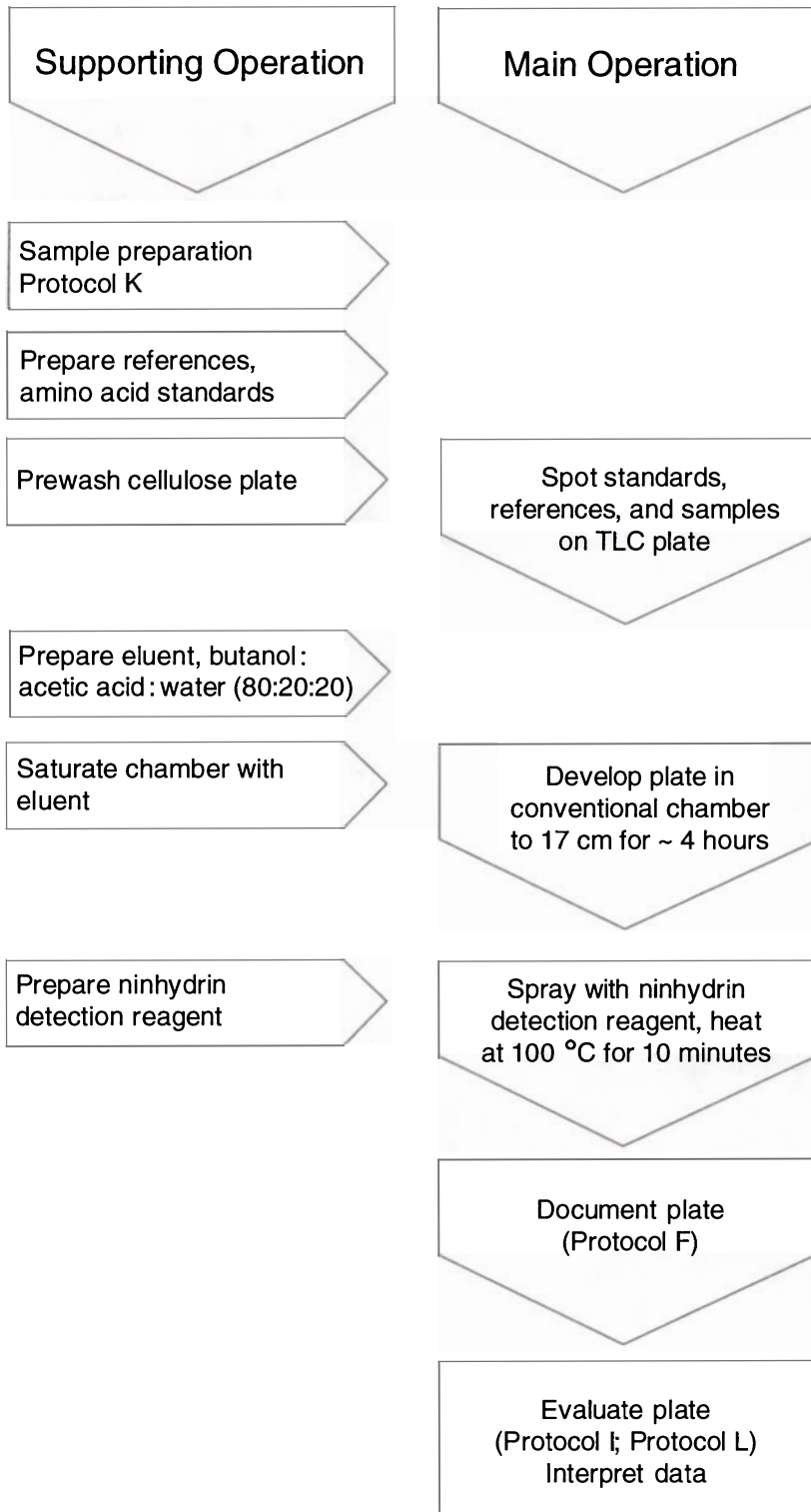# Identification of Proteins by Thin-Layer Chromatography

**Summary**

Separation of amino acids from hydrolysates for the identification of proteins

| | |
|---|---|
| Layer: | Macherey-Nagel polyester-backed cellulose |
| Eluent: | Butanol : acetic acid : water (80:20:20) |
| Technique: | Single development, conventional chamber |
| Migration Distance: | 17 cm in approximately 4 hours |
| Detection: | Spray plates evenly with 0.2% ninhydrin detection reagent; heat at 100 °C for 10 minutes; document after 24 hours |

**Scope**

Identification of proteins by thin-layer chromatography is based on the presence or absence of certain amino acids within the protein. For instance, the presence of large quantities of hydroxyproline is indicative of an animal glue, and casein is identified in a sample by the presence of a larger concentration of proline and the absence of hydroxyproline. Further identification can be performed through computer analysis of the plate (see **Protocol L**).

First, the sample and reference materials are hydrolyzed into their component amino acids (see **Protocol K**). Then the sample, individual amino acid standards, and reference materials are spotted onto a cellulose TLC plate. The plate is developed in a conventional TLC chamber with a butanol, acetic acid, and water solvent system. The development of the plate takes approximately 4 hours. The amino acids are visualized by spraying the TLC plate with a ninhydrin detection reagent.

**Scheme**

**Protein Analysis by Thin-Layer Chromatography**

| Supporting Operation | Main Operation |
|---|---|

Sample preparation
Protocol K

Prepare references,
amino acid standards

Prewash cellulose plate

Spot standards,
references, and samples
on TLC plate

Prepare eluent, butanol:
acetic acid: water (80:20:20)

Saturate chamber with
eluent

Develop plate in
conventional chamber
to 17 cm for ~ 4 hours

Prepare ninhydrin
detection reagent

Spray with ninhydrin
detection reagent, heat
at 100 °C for 10 minutes

Document plate
(Protocol F)

Evaluate plate
(Protocol I; Protocol L)
Interpret data

## 1. Equipment and Supplies

The following equipment is needed for a single development, conventional TLC analysis:
1) Amber glass storage bottles (250 ml)
2) Capillary pipettes (1.0 and 0.2 µl size)
3) Conventional TLC chamber with a lid
4) Glass vials with caps (1 and 4 ml)
5) Graduated cylinder (100 ml)
6) Oven
7) Reagent sprayer
8) Ruler (inch and metric)
9) Saturation pad (20 x 20 cm)
10) Spray box
11) Spray stand

## 2. Chemicals and Materials

1) 0.1*N* hydrochloric acid
2) Eluent components
   *Butanol*
   *Acetic acid*
   *Water*
3) Ethanol
4) Macherey-Nagel cellulose plate, 20 x 20 cm
5) Methanol
6) Ninhydrin (**Caution: toxic reagent, handle with care**)
7) Amino acid standard solutions (1 mg/ml)
   *Glutamic acid*   *Hydroxyproline*
   *Lysine*          *Proline*
   *Serine*          *Threonine*
   *Tyrosine*
8) Binding media reference materials (hydrolyzed)
   *Whole egg*       *Egg white*
   *Egg yolk*        *Casein*
   *Rabbit-skin glue*  *Fish glue*

## 3. Samples

Samples may be taken from facsimile paintings or unknowns. The sample should be approximately 500 µg in weight and contain only the paint layer or material of interest. The paint layer or material being investigated should be separated from all other layers, such as the ground, varnish layers, or support. Samples are hydrolyzed before analysis, following **Protocol K**.

Amino acid standard solutions are made with glutamic acid, hydroxyproline, lysine, proline, serine, threonine, and tyrosine. Each standard solution is made in a

concentration of 1 mg/ml by weighing 2 mg of an amino acid into a 4-ml glass
vial and adding 2 ml of 0.1N HCI. These solutions can be used for 3–4 weeks
after preparation.

Reference solutions of binding media are made from whole egg, egg white,
egg yolk, casein, rabbit-skin glue, and fish glue. These solutions are prepared by
hydrolysis following the same procedure as for the samples (**Protocol K**).
The reference materials should be prepared in a concentration of 2.0–2.5 µg/µl
in 0.1N HCI.

## 4. Preparation Procedures

Preparation for TLC analysis includes prewashing the TLC plate, making fresh
eluent systems and detection reagents, and saturating the TLC chamber.
The following preparation procedures are started 24 hours prior to analysis:

1) **Prepare cellulose TLC plates**
   The cellulose plate must be washed in methanol before analysis. This
   procedure takes approximately 4 hours. Place 30–60 ml of methanol in a
   clean conventional TLC chamber. Allow the chamber to equilibrate
   with methanol for approximately 30 minutes. The cellulose TLC plate is
   inserted vertically into the methanol, and the chamber is covered with the
   lid. Allow the methanol to rise to the top of the cellulose TLC plate. Remove
   the plate from the chamber and dry it in a fume hood. Store the cleaned
   cellulose TLC plate in a desiccator containing silica gel.

2) **Prepare eluent**
   Mix butanol, acetic acid, and water in an 80:20:20 volume ratio. Seal the
   solution in an amber bottle to maintain freshness before use. Prepare 60 ml
   of the eluent fresh daily for an analysis.

3) **Prepare TLC chamber**
   Presaturate chamber with solvent system at least 4 hours before analysis.
   (Note: It is useful to presaturate the chamber overnight.) To do this, place
   30–60 ml of the eluent inside a clean, dry conventional TLC chamber. Insert a
   saturation pad into the solvent system. Cover the chamber with a lid.

4) **Prepare ninhydrin detection reagent**
   Weigh out 0.158 g of ninhydrin into a 250-ml amber bottle. Add 100 ml of
   ethanol. Mix thoroughly. The reagent can be stored in a refrigerator
   for 4–5 weeks.

## 5. TLC Analysis Procedures

To analyze protein hydrolysates by TLC, the samples are spotted in individual
lanes at the baseline of a prewashed cellulose plate. The plate is placed in a satu-
rated conventional TLC chamber containing a saturation pad and the eluent
(butanol : acetic acid : water, 80:20:20). The development of the plate is complete
when the eluent front reaches a distance of 17 cm from the baseline. The plate is
removed and dried in a fume hood before spraying with the ninhydrin reagent.
This reagent reacts with the amino acid components to produce colors that aid in

the visualization of the separation zones or spots. After 24 hours the plate can be documented.

The following nine steps describe the procedure for analysis:

1) **Draw the baseline**

   Using a ruler and pencil, lightly draw a line 1 cm from the bottom edge of the plate. Very lightly mark the lanes with short tick marks at intervals of 1 cm along this baseline, for a total of 19 lanes. In the upper left corner, number the plate with a reference number, used to relate the TLC to information in the research notes. Beside the number, place the date and the analyst's initials. Place a mark 17 cm from the baseline as a reference to help determine the completion of the development.

2) **Apply the standard and reference solutions to the plate**

   All solutions are applied following the spotting procedure noted in **Protocol H**. Apply 1.0 μl of the reference or standard solution to a tick mark on the origin of a lane using a capillary pipette. The total volume may be applied in a series of smaller volumes to minimize the diameter of the spot. An air gun may be used to rapidly evaporate the carrier solvent between applications. *Take care not to get the air gun too close to the pipette, as the sample will evaporate.*

3) **Apply unknown sample solutions**

   If possible, apply each unknown sample in two different volumes. For example, in one lane apply 1.0 μl of the unknown sample, and in a second lane apply 0.2 μl of the same solution. (The unknown sample may or may not be very concentrated, and this procedure minimizes the possibility of overloading the plate.)

4) **Develop the TLC plate**

   Once the plate is spotted, either develop immediately or store in a desiccator. To develop the plate, quickly insert the spotted cellulose TLC plate into the saturated chamber, with the baseline oriented toward the bottom of the chamber and the front facing away from the saturation pad. Replace the lid of the chamber. *Do not leave the chamber open for any length of time, as the vapor phase equilibrium will be lost.*
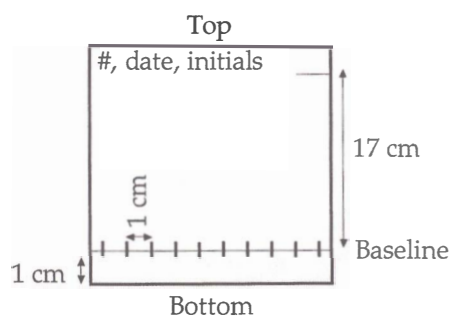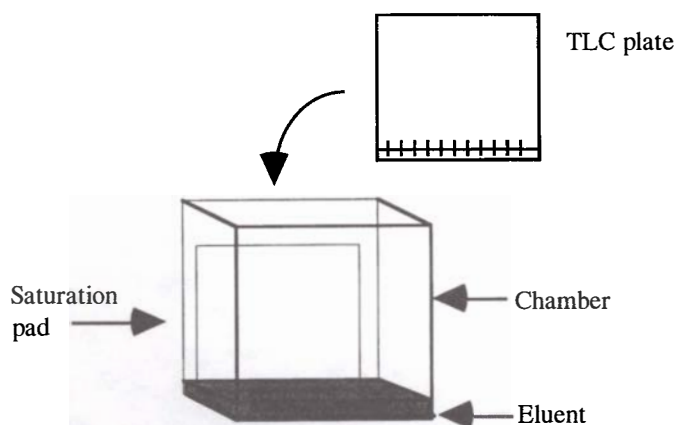
5) **Completion of development**
   Develop the plate until the solvent front travels a distance of 17 cm. Development usually takes about 4 hours.
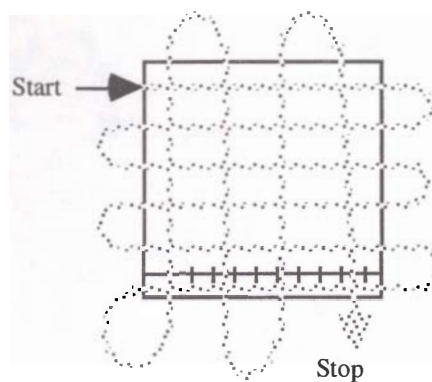
6) **Dry the plate**
   Remove the plate from the chamber, hang it vertically, and let it dry for about 30 minutes at room temperature in the fume hood.

7) **Prepare to spray plate**
   Spraying of a ninhydrin reagent should always be performed under a well-ventilated fume hood or some other device to ensure effective removal of the reagent cloud and solvent vapors, which are toxic. Protective glasses, laboratory gloves, and a respirator should always be worn during spraying. Set the plate on a clean, dry spray stand inside a spray box. Fill the reagent sprayer with 15–20 ml of ninhydrin detection reagent.

8) **Spray plate with ninhydrin**
   Hold the reagent sprayer 8–10 cm from the surface of the TLC plate and spray the plate slowly back and forth, then up and down, until the plate is evenly covered (generally until the cellulose layer just begins to turn transparent).



9) **Heat plate**
   Dry the plate for 15–20 minutes in the fume hood, then place it for 10 minutes in a preheated oven at 100 °C.

## 6. Data Analysis Procedures

After the separation zones are visualized with the detection reagent, the plate is documented (see **Protocol F**) and evaluated (see **Protocol I**). Documentation includes two procedures. The first is written documentation that includes annotation in a bound research notebook of all methods and procedures used. The second is visual documentation, which includes making a photocopy of the TLC plate (to be placed in the notebook) and photographing or digitally scanning the plate. Evaluation of the plate can include qualitative or semiquantitative techniques. The migration distances, color, and intensity of the separation spots are noted. The $R_f$ value for each spot is calculated.

## 7. Interpretation

The chromatographic pattern of the unknown samples is compared to those of the reference materials run on the same plate. Identification of the unknown sample can be made if its chromatographic pattern closely matches that of a reference sample. Also, the presence or absence of certain amino acids in the chromatogram aids in identification of the unknown. Animal glues can be identified by the presence of hydroxyproline. However, different types of animal glue cannot be distinguished by the chromatographic pattern. Casein can be identified by the presence of larger concentrations of proline and the absence of hydroxyproline. Distinctions between egg white, egg yolk, and whole egg may not be readily obvious in the chromatographic patterns when they are examined by eye.

Protocol B

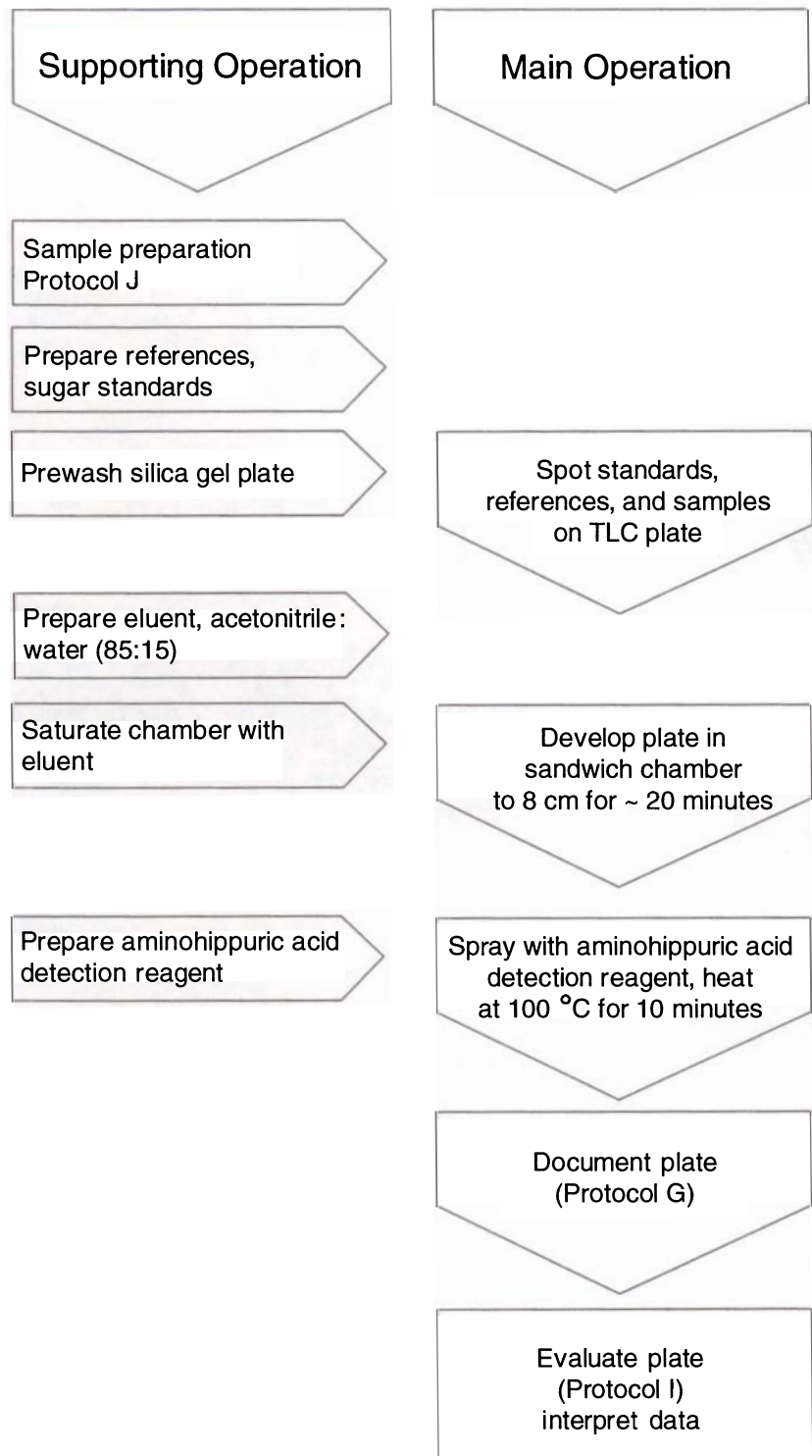# Identification of Carbohydrates by Thin-Layer Chromatography

**Summary**

Separation of monosaccharides from hydrolysates for the identification of carbohydrates

| | |
|---|---|
| Layer: | Merck HPTLC silica gel 60 |
| Eluent: | Acetonitrile : water (85:15) |
| Technique: | Single development, sandwich chamber |
| Migration Distance: | 8 cm in approximately 20 minutes |
| Detection: | Spray plates evenly with 0.3% solution of aminohippuric acid with 3% phthalic acid in ethanol; heat at 100 °C for 10 minutes; document within 24 hours |

**Scope**    Carbohydrate media are complex polysaccharides that can be identified by the presence or absence of certain simple sugars (monosaccharides) within the hydrolyzed sample. First, the sample and reference materials are hydrolyzed into their component monosaccharides (see **Protocol J**). Then the sample, individual sugar standards, and reference materials are spotted on a prepared silica gel HPTLC plate. The plate is developed in a sandwich TLC chamber using an acetonitrile : water solvent system. The development of the plate takes approximately 20 minutes. The sugars are visualized by spraying the TLC plate with an aminohippuric acid detection reagent.

**Scheme**

**Carbohydrate Analysis by Thin-Layer Chromatography**

| Supporting Operation | Main Operation |
|---|---|
| Sample preparation Protocol J | |
| Prepare references, sugar standards | |
| Prewash silica gel plate | Spot standards, references, and samples on TLC plate |
| Prepare eluent, acetonitrile: water (85:15) | |
| Saturate chamber with eluent | Develop plate in sandwich chamber to 8 cm for ~ 20 minutes |
| Prepare aminohippuric acid detection reagent | Spray with aminohippuric acid detection reagent, heat at 100 °C for 10 minutes |
| | Document plate (Protocol G) |
| | Evaluate plate (Protocol I) interpret data |

## 1. Equipment and Supplies

The following equipment is needed for a single development TLC analysis using a sandwich chamber:

1) Amber glass storage bottles (250 ml)
2) Capillary pipettes (1.0 and 0.2 μl size)
3) Hot plate
4) Thermometer
5) Cylindrical TLC chamber and lid
6) Sandwich chamber
7) Glass vials with Teflon septum caps (4 ml)
8) 250-ml vial inserts and compression springs
9) Graduated cylinder (100 ml)
10) Oven
11) Reagent sprayer
12) Ruler (inch and metric)
13) Spray box
14) Spray stand

## 2. Chemicals and Materials

1) 0.1*N* hydrochloric acid
2) Eluent components
   *Acetonitrile*
   *Water*
3) Merck HPTLC silica gel 60 plate, 10 x 10 cm
4) Methanol
5) Ethanol
6) Aminohippuric acid
7) Phthalic acid
8) Sugar standard solutions (1 mg/ml)

   | *Arabinose* | *Xylose* |
   |---|---|
   | *Galactose* | *Glucuronic acid* |
   | *Glucose* | *Galacturonic acid* |
   | *Mannose* | *Ribose* |
   | *Rhamnose* | *Fucose* |
   | *Mixed standard* | |

9) Binding media reference materials (hydrolyzed)

   | *Gum arabic* | *Gum ghatti* |
   |---|---|
   | *Gum tragacanth* | *Guar gum* |
   | *Cherry gum* | |

## 3. Samples

Samples can be taken from facsimile paintings or unknowns. The sample should be approximately 500 μg in weight and should contain only the paint layer or material of interest. The paint layer or material being investigated should be sepa-

rated from all other layers, such as the ground, varnish layers, or support. Samples are hydrolyzed before analysis, following **Protocol J**.

Sugar standard solutions are made from arabinose, rhamnose, galactose, glucose, mannose, xylose, fucose, glucuronic acid, galacturonic acid, and ribose. Each standard solution is made in a concentration of 1 mg/ml by weighing 3 mg of a sugar into a 4-ml glass vial and adding 3 ml of methanol. A mixed standard is prepared by weighing 3 mg of each of the sugars into a 4-ml vial and adding 3 ml of methanol. These solutions can be used for 3–4 weeks after preparation.

Reference solutions of binding media are made from gum arabic, gum tragacanth, cherry gum, gum ghatti, and guar gum. The reference solutions of binding media are made at a concentration of 1.25 mg/ml in methanol. These solutions are prepared by hydrolysis following the same procedure as for the samples (**Protocol J**).

## 4. Preparation Procedures

Preparation for TLC analysis includes prewashing the TLC plate and making fresh eluent systems and detection reagents. The following preparation procedures are started 24 hours prior to analysis:

1) **Prepare silica TLC plates**

   The silica gel plate is washed with methanol, then heated before use. This procedure takes about 2 hours. The plate can be washed in a sandwich chamber or a conventional chamber. The procedure described here uses a sandwich chamber.

   Place 5 ml of methanol in the trough of a TLC sandwich chamber. Position the silica gel plate so that the stationary phase faces the backing plate, and adjust the spacing with the spacing guide. Attach the silica plate to the backing plate with two clips. Make sure the clips are attached near the top of the plate. Insert the assembly vertically into the trough containing the methanol. Place the assembly inside a cylindrical TLC chamber and cover with the lid. Allow the methanol to rise to the top of the silica gel plate. This process usually takes 20 minutes. Remove the plate from the chamber and dry it in a fume hood. Place the plate in an oven at 100 °C for 1 hour to activate the silica gel. After cooling, store the clean, activated silica plate in a desiccator containing silica gel. The plate is ready for use after it cools (usually 30 minutes after it is removed from the oven).
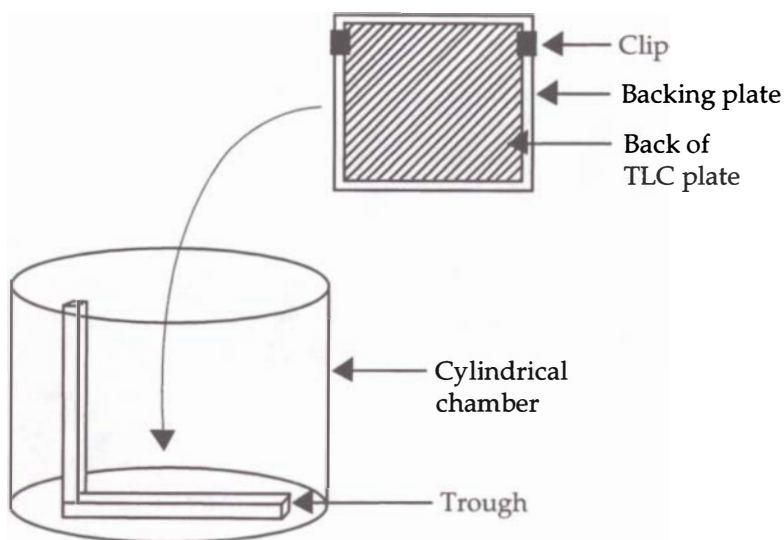
2) **Prepare eluent**

Prepare 60 ml of the eluent fresh daily for an analysis. Mix acetonitrile and water in an 85:15 ratio. Seal the solution in an amber bottle to maintain freshness.

3) **Prepare cylindrical chamber**

Presaturate the cylindrical TLC chamber with 10 ml of the solvent system at least 30 minutes before analysis. Place the trough of the sandwich chamber inside the cylindrical chamber and cover it with a lid.
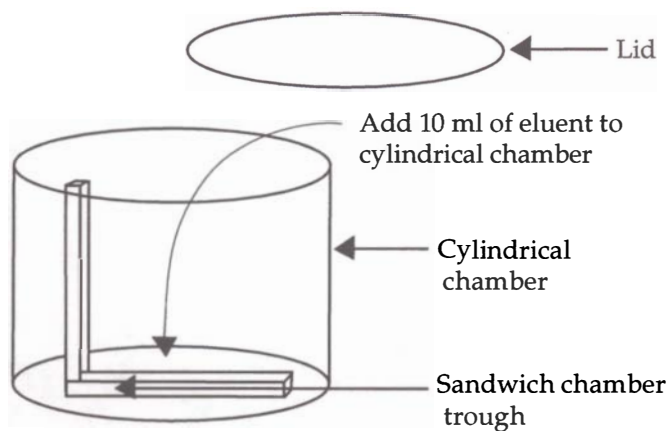


4) **Prepare aminohippuric acid detection reagent**

The detection reagent is a 0.3% solution of aminohippuric acid with 3% phthalic acid in ethanol. Weigh out 0.24 g of aminohippuric acid and 2.4 g of phthalic acid into a 250 ml amber bottle. Add 100 ml of ethanol. Mix thoroughly until all solids are dissolved. This reagent can be stored for 2–3 weeks.
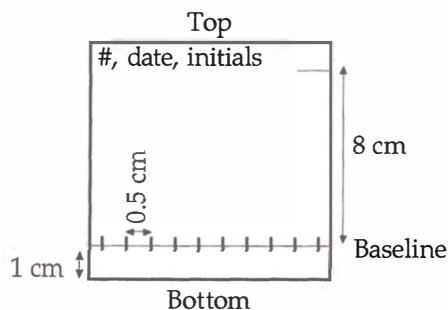
## 5. TLC Analysis Procedures

To analyze carbohydrates by TLC, the hydrolysates of samples are spotted in individual lanes at the baseline of a prewashed, activated HPTLC silica gel plate. The plate is attached to the backing plate of a sandwich chamber and inserted into a small trough containing the eluent (acetonitrile : water, 85:15). The development of the plate takes place inside a cylindrical TLC chamber to reduce the effects of air currents on the movement of the solvent front. This also minimizes the premature evaporation of the solvent system. The development of the plate is complete when the eluent front travels a distance of 8 cm from the baseline. The plate is removed and dried in a fume hood before spraying with the aminohippuric acid reagent. Patterns of fluorescent spots are visible for each sample when the plate is viewed under ultraviolet light. The plate may be photographed for documentation immediately.

The following nine steps describe the procedure for analysis:

1) **Draw the baseline**

    Using a ruler and pencil, lightly draw a line 1 cm from the bottom edge of the plate. Very lightly mark the lanes with short tick marks at intervals of 0.5 cm along this baseline, for a total of 19 lanes. In the upper left corner, number the plate with a reference number, used to relate the TLC to information in the research notes. Beside the number, place the date and the operator's initials. Place a mark 8 cm from the baseline as a reference to help determine the completion of the development. If this mark is scored heavily through the silica gel, it will be visible on both sides of the plate.



2) **Apply the standard and reference solutions to the plate**

    All solutions are applied following the spotting procedure noted in **Protocol H**. Apply 1.0 µl of the reference or standard solution to a tick mark on the origin of a lane using a capillary pipette. The total volume may be applied in a series of smaller volumes to minimize the diameter of the spot. An air gun may be used to rapidly evaporate the carrier solvent between applications. *Take care not to get the air gun too close to the pipette, as the sample will evaporate.*

3) **Apply unknown sample solutions**

    If possible, apply each unknown sample in two different volumes. For example, in one lane apply 1.0 µl of the unknown sample, and in a second lane apply 0.2 µl of the same solution. (The unknown sample may or

may not be very concentrated, and this procedure minimizes the possibility of overloading the plate.)

4) **Develop the TLC plate**

Once the plate is spotted, develop immediately or store in a desiccator. Open the lid of the cylindrical chamber slightly and quickly place 5 ml of eluent in the trough of the TLC sandwich chamber, located inside the cylindrical chamber. Position the face of the silica gel plate toward the backing plate and adjust the spacing between the edge of the plate and the rim of the backing plate, using the spacing guide. Attach the silica plate to the backing plate with two clips. Make sure that the clips are attached near the top of the plate, next to the spacers. Quickly open the chamber and set the bottom edge of this assembly into the trough containing the eluent. Replace the lid of the chamber. *Do not leave the cylindrical chamber open for any length of time, as the vapor phase equilibrium will be lost.*
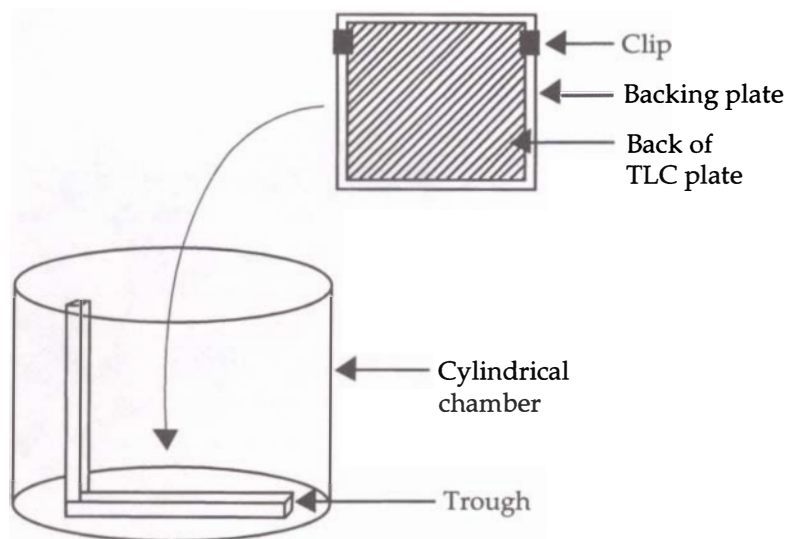


5) **Completion of development**

Develop the plate until the solvent front reaches the tick mark at 8 cm. Development usually takes about 20 minutes.

6) **Dry the plate**

Remove the plate from the chamber and let it dry for about 30 minutes at room temperature in the fume hood.

7) **Prepare to spray plate**

Spraying of an aminohippuric acid reagent should always be performed under a well-ventilated fume hood or some other device to ensure effective removal of the reagent cloud and solvent vapors. Protective glasses, laboratory gloves, and a respirator should always be worn during spraying. Set the plate on a clean, dry spray stand inside a spray box. Fill the reagent sprayer with 15–20 ml of aminohippuric acid detection reagent.

8) **Spray plate with aminohippuric acid**

Hold the reagent sprayer 8–10 cm from the surface of the TLC plate and spray the plate slowly back and forth, then up and down, until the plate is evenly covered (generally until the silica gel layer just begins to turn transparent).
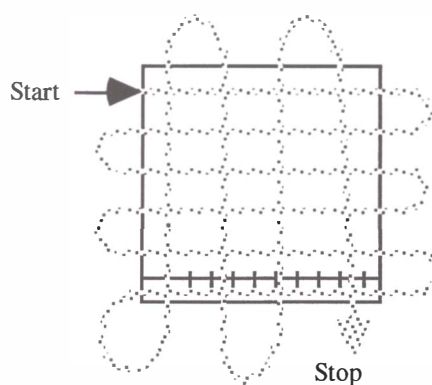
9) **Heat plate**

Dry the plate for 15–20 minutes in the fume hood, then place it in a preheated oven at 100 °C for 10 minutes.

## 6. Data Analysis Procedures

After detection by derivatization, the plate is documented (see **Protocol G**) and evaluated (see **Protocol I**). Documentation includes two procedures. The first is written documentation that includes annotation in a bound research notebook of all methods and procedures used. The second is visual documentation, which includes making a photocopy of the TLC plate (to be placed in the notebook) and photographing or digitally scanning the plate. Evaluation of the plate can include qualitative or semiquantitative techniques. The migration distances, color, and intensity of the separation spots are noted. The $R_f$ value for each spot is calculated.

## 7. Interpretation

The chromatographic pattern of the unknown materials is compared to those of the reference materials run on the same plate. Identification of the unknown sample can be made if its chromatographic pattern closely matches that of a reference sample.

This chromatographic system allows complete separation of galacturonic acid, glucuronic acid, galactose, arabinose, and rhamnose. Other simple sugars, such as xylose, fucose, and ribose, or glucose and mannose, are not completely resolved. The ascending order of $R_f$ values for sugars is galacturonic acid < glucuronic acid < galactose < glucose ≈ mannose < arabinose < fucose ≈ xylose ≈ ribose < rhamnose. All reference gum samples (i.e., gum tragacanth, gum arabic, etc.) give distinguishable chromatographic patterns. However, dextrin and wheat starch cannot be differentiated on the basis of their chromatograms.

Protocol C

# Identification of Waxes by Thin-Layer Chromatography

**Summary**

Separation of chloroform extracts for the identification of waxes

| | |
|---|---|
| Layer: | Merck HPTLC silica gel |
| Eluent: | Petroleum ether : ethyl ether : acetic acid (90:10:1) |
| Technique: | Single development, sandwich chamber |
| Migration Distance: | 8 cm in approximately 15 minutes |
| Detection: | Spray plates evenly with anisaldehyde solution; heat at 100 °C for 10 minutes; document immediately after detection |

**Scope**

Identification of waxes by thin-layer chromatography is based on comparison of the chromatographic patterns for reference and unknown materials. The number, location, and color of the spots are noted.

Wax samples are dissolved in chloroform and the soluble portion is spotted onto a prewashed HPTLC silica gel plate. The plate is developed in a sandwich chamber containing an eluent made from petroleum ether, ethyl ether, and acetic acid. The components of each wax are visualized by spraying with an anisaldehyde detection reagent. The plate is heated after spraying to activate the reagent, then examined under ultraviolet light. The components are seen as fluorescent spots.

## Scheme

## Wax Analysis by Thin-Layer Chromatography
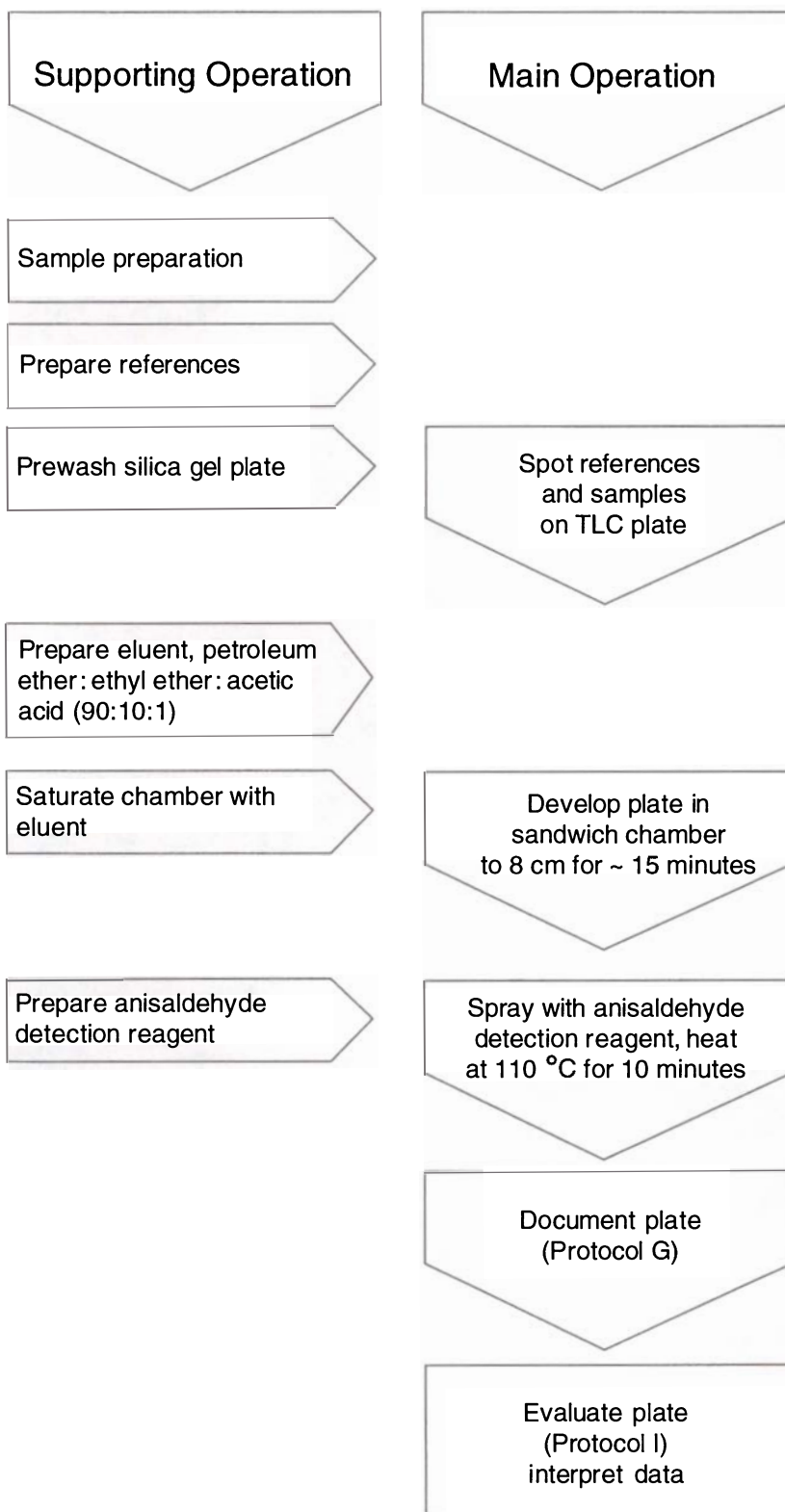
| Supporting Operation | Main Operation |
|---|---|

Sample preparation

Prepare references

Prewash silica gel plate

Spot references
and samples
on TLC plate

Prepare eluent, petroleum
ether : ethyl ether : acetic
acid (90:10:1)

Saturate chamber with
eluent

Develop plate in
sandwich chamber
to 8 cm for ~ 15 minutes

Prepare anisaldehyde
detection reagent

Spray with anisaldehyde
detection reagent, heat
at 110 °C for 10 minutes

Document plate
(Protocol G)

Evaluate plate
(Protocol I)
interpret data

## 1. Equipment and Supplies

The following equipment is needed for a single development TLC analysis using a sandwich chamber:

1) Amber glass storage bottles (250 ml)
2) Capillary pipettes (1.0 and 0.2 μl size)
3) Hot plate
4) Thermometer
5) Cylindrical TLC chamber and lid
6) Sandwich chamber
7) Glass vials with Teflon septum caps (4 ml)
8) 250 μl vial inserts and compression springs
9) Graduated cylinder (100 ml)
10) Oven
11) Reagent sprayer
12) Ruler (inch and metric)
13) Spray box
14) Spray stand

## 2. Chemicals and Materials

1) Chloroform
2) Eluent components
   - *Petroleum ether*
   - *Ethyl ether*
   - *Acetic acid*
3) Merck HPTLC silica gel plate, 10 x 10 cm
4) Methanol
5) Anisaldehyde detection reagent
   - *Anisaldehyde* **(Caution: handle with care)**
   - *Acetic acid*
   - *Methanol*
   - *Concentrated sulfuric acid*
6) Wax reference materials in chloroform (10 mg/ml)

| | |
|---|---|
| *Candelilla wax* | *Paraffin wax* |
| *Carnauba wax* | *Rice wax* |
| *Ceresine wax* | *Spermaceti wax* |
| *Earth wax* | *Bleached beeswax* |
| *Japan wax* | *White beeswax* |
| *Montan wax* | *Yellow beeswax* |

## 3. Samples

Samples can be taken from facsimile objects or unknowns. The sample should be approximately 500 μg in weight and contain only the paint layer or material of interest. The paint layer or material being investigated should be separated from all other layers, such as the ground, varnish layers, or support. Samples are placed

in a 250-µg insert vial and are dissolved in 80 µl chloroform. Some of the sample may not totally dissolve in the chloroform solution.

Reference solutions of waxes are made in a concentration of 10 mg/ml from candelilla, carnauba, ceresine, earth, Japan, montan, paraffin, rice, and spermaceti wax, as well as bleached, white, and yellow beeswax. These solutions are prepared by weighing 800 µl of wax into a 250-µg insert vial and adding 80 µl of chloroform. The insert vial is placed inside a compression spring, and the entire unit is placed in a 4-ml glass vial. This vial is then sealed with the Teflon septum cap.
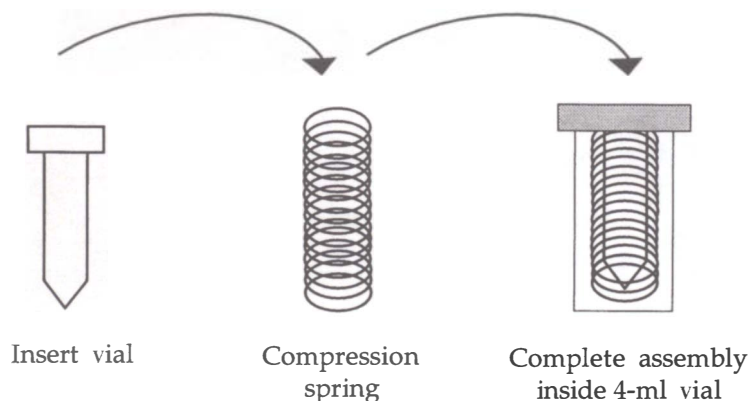


Insert vial      Compression      Complete assembly
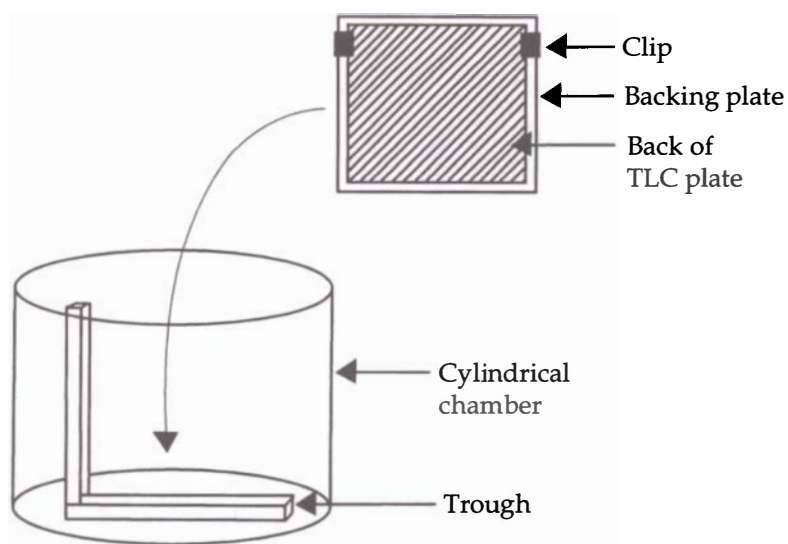                 spring            inside 4-ml vial

## 4. Preparation Procedures

Preparation for TLC analysis includes prewashing the TLC plate and making fresh eluent systems and detection reagents. The following preparation procedures are started 24 hours prior to analysis:

1) **Prepare silica TLC plates**

   The silica gel plate is washed with methanol, then heated before use. This procedure takes about 2 hours. The plate can be washed in a sandwich chamber or a conventional chamber. The procedure described here uses a sandwich chamber.

   Place 5 ml of methanol in the trough of a TLC sandwich chamber. Position the silica gel plate so that the stationary phase faces the backing plate and adjust the spacing using the spacing guide. Attach the silica plate to the backing plate with two clips. Make sure that the clips are attached near the top of the plate. Insert the assembly vertically into the trough containing the methanol. Place all parts inside a cylindrical TLC chamber and cover with the lid. Allow the methanol to rise to the top of the silica gel plate. This process usually takes 20 minutes. Remove the plate from the chamber and dry it in a fume hood. Place the plate in an oven at 100 °C for 1 hour to activate the silica gel. Cool and store the clean, activated silica plate in a desiccator containing silica gel. The plate is ready for use after it cools (usually 30 minutes after it is removed from the oven).

2) **Prepare eluent**

Prepare 60 ml of the eluent fresh daily for an analysis. Mix petroleum ether, ethyl ether, and acetic acid in a 90:10:1 ratio. Seal the solution in an amber bottle to maintain freshness.

3) **Prepare cylindrical chamber**

Presaturate the cylindrical TLC chamber with 10 ml of the solvent system at least 30 minutes before analysis. Place the trough of the sandwich chamber inside the cylindrical chamber. Cover the cylindrical chamber with a lid.
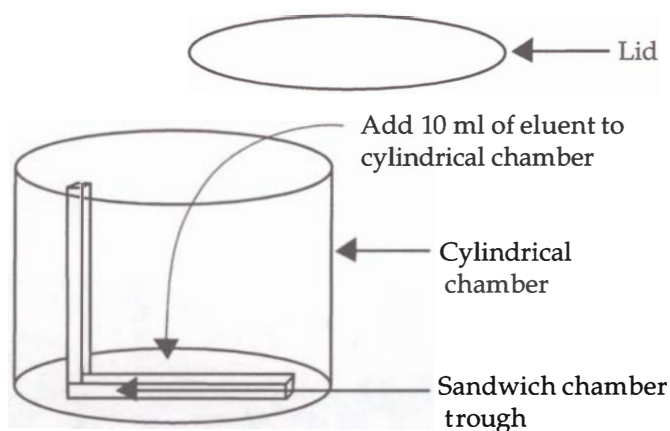


4) **Prepare anisaldehyde detection reagent**

Measure 0.5 ml of anisaldehyde into a 250-ml amber bottle. Add 10 ml of concentrated acetic acid, 84.5 ml of methanol, and 5 ml of concentrated sulfuric acid. Mix thoroughly. The reagent can be stored for 1–2 weeks.

**Reminder: Highly corrosive concentrated acetic acid and sulfuric acid are used in this detection reagent. They should be handled with extremecare in a well-ventilated fume hood. A lab coat, goggles, and protective gloves should be worn when handling. Avoid contact with liquid or vapors.**

## 5. TLC Analysis Procedures
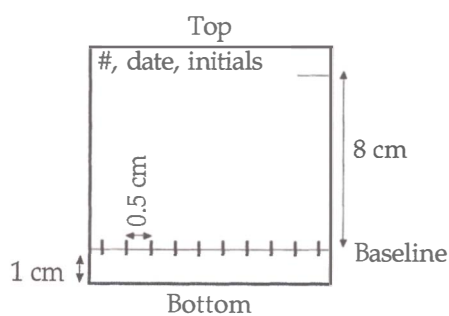
To analyze waxes by TLC, the chloroform extracts of samples are spotted in individual lanes at the baseline of a prewashed, activated HPTLC silica gel plate. The plate is attached to the backing plate of a sandwich chamber and inserted into a small trough containing the eluent (petroleum ether : ethyl ether : acetic acid, 90:10:1). The development of the plate takes place inside a cylindrical TLC chamber to minimize the effects of air currents on the movement of the solvent front. This also minimizes the premature evaporation of the solvent system. The development of the plate is complete when the eluent front reaches a distance of 8 cm from the baseline. The plate is removed and dried in a fume hood before spraying with the anisaldehyde reagent. Patterns of fluorescent spots are visible for each sample when the plate is viewed under ultraviolet light. The plate may be photographed for documentation immediately.

The following nine steps describe the procedure for analysis:

1) **Draw the baseline**

   Using a ruler and pencil, lightly draw a line 1 cm from the bottom edge of the plate. Very lightly mark the lanes with short tick marks at intervals of 0.5 cm along this baseline, for a total of 19 lanes. In the upper left corner, number the plate with a reference number, used to relate the TLC to information in the research notes. Beside the number, place the date and the operator's initials. Place a mark 8 cm from the baseline as a reference to help determine the completion of the development. If this mark is scored heavily through the silica gel, it will be visible on both sides of the plate.



2) **Apply the reference solutions to the plate**

   All solutions are applied following the spotting procedures noted in **Protocol H.** Approximately 10 minutes before analysis, gently heat the reference solutions to 40 °C on a hot plate. Apply 0.5 µl of each reference solution on a tick mark at the origin of a lane, using a capillary pipette. The total volume may be applied in a series of smaller volumes to minimize the diameter of the spot. An air gun may be used to rapidly evaporate the carrier solvent between applications. *Take care not to get the air gun too close to the pipette, as the sample will evaporate.*

3) **Apply unknown sample solutions**

   Again, heat the solutions to 40 °C 10 minutes before application. If possible, apply each unknown sample in two different volumes. For example, in one

lane apply 1.0 µl of the unknown sample, and in a second lane apply 0.2 µl of the same solution. (The unknown sample may or may not be very concentrated, and this procedure minimizes the possibility of overloading the plate.)

4) **Develop the TLC plate**
Once the plate is spotted, develop immediately or store in a desiccator. Open the lid of the cylindrical chamber slightly, and quickly place 5 ml of eluent in the trough of a TLC sandwich chamber, located inside the cylindrical chamber. Position the silica gel plate so the stationary phase faces the backing plate and adjust spacing between the edge of the plate and the rim of the backing plate with the spacing guide. Attach the silica plate to the backing plate with two clips. Make sure that the clips are attached near the top of the plate, next to the spacers. Quickly open the cylindrical chamber and set the bottom edge of this assembly into the trough containing the eluent. Replace the lid of the chamber. *Do not leave the chamber open for any length of time, as the vapor phase equilibrium will be lost.*
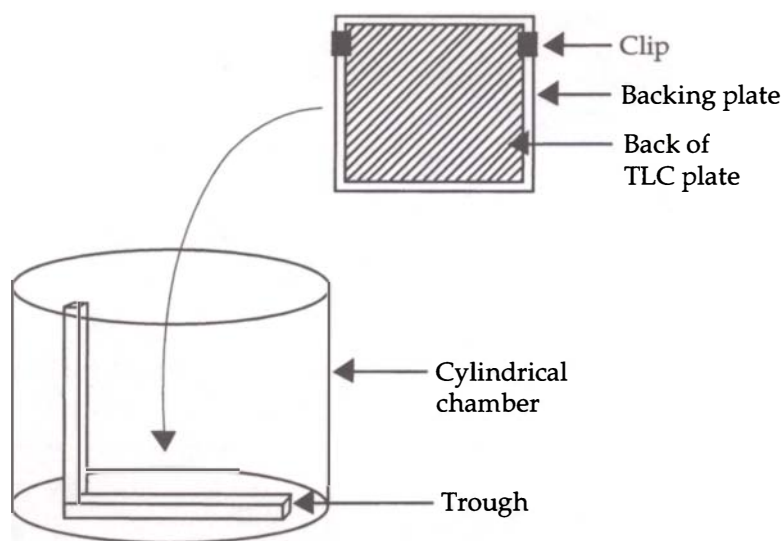


5) **Completion of development**
Develop the plate for a distance of 8 cm. Development usually takes about 15 minutes.

6) **Dry the plate**
Remove the plate from the chamber and let it dry for about 30 minutes at room temperature in the fume hood.

7) **Prepare to spray plate**
**Spraying of an anisaldehyde reagent should always be performed under a well-ventilated fume hood or some other device to ensure effective removal of the reagent cloud and solvent vapors, which are corrosive and toxic. Protective glasses, laboratory gloves, and a respirator should always be worn during spraying.** Set the plate on a clean, dry spray stand inside a spray box. Fill the reagent sprayer with 15–20 ml of anisaldehyde detection reagent.

8) **Spray plate with anisaldehyde**

 Hold the reagent sprayer 8–10 cm from the surface of the TLC plate and spray the plate slowly back and forth, then up and down, until the plate is evenly covered. This is usually when the thin layer of silica gel is wetted with just enough detection reagent to begin to turn transparent.



9) **Heat plate**

 Dry the plate for 15–20 minutes in the fume hood, then place it in a preheated oven at 100 °C for 10 minutes.

## 6. Data Analysis Procedures

After the separation zones are visualized by reaction with the detection reagent, the plate is documented (see **Protocol G**) and evaluated (see **Protocol I**). Documentation includes two procedures. The first is written documentation that includes annotation in a bound research notebook of all methods and procedures used. The second is visual documentation, which includes making a photocopy of the TLC plate (to be placed in the notebook) and photographing or digitally scanning the plate. Evaluation of the plate can include qualitative or semiquantitative techniques. The migration distances, color, and intensity of the separation spots are noted. The $R_f$ value for each spot is calculated.

## 7. Interpretation

The chromatographic pattern of the unknown materials is compared to those of the reference materials run on the same plate. Identification of the unknown sample can be made if its chromatographic pattern closely matches that of a reference sample.

Identification of the individual separation zones of each wax is not attempted. Separation of at least nine spots is seen for three types of beeswax (white, yellow, and bleached). All other waxes give distinguishable chromatographic patterns.

Protocol D

# Identification of Resins by Thin-Layer Chromatography

**Summary**

Separation of ethyl acetate extracts for the identification of resins

| | |
|---|---|
| Layer: | Merck HPTLC silica gel |
| Eluent: | Benzene : methanol (95:5) |
| Technique: | Threefold development, sandwich chamber |
| Migration Distance: | 8 cm in approximately 30 minutes (3 times; total time = 1.5 hours) |
| Detection: | Spray plates evenly with antimony trichloride detection reagent; heat at 100 °C for 10 minutes; document immediately after detection |

**Scope**　Identification of resins by thin-layer chromatography is based on comparison of the chromatographic patterns for reference and unknown materials. The number, location, and color of the spots are noted.

The resin samples are dissolved in ethyl acetate. The soluble portion of the resin is applied to a prewashed, activated HPTLC silica gel plate. The plate is developed in a sandwich chamber, using a benzene and methanol eluent. The chromatographic plate is usually developed two or three times in the same solvent system to improve resolution of the spots in the chromatographic patterns. The plate is sprayed with a commercially available antimony trichloride detection reagent. Examination of the plate under ultraviolet light reveals a variety of colored fluorescent spots.

## Scheme

## Resin Analysis by Thin-Layer Chromatography
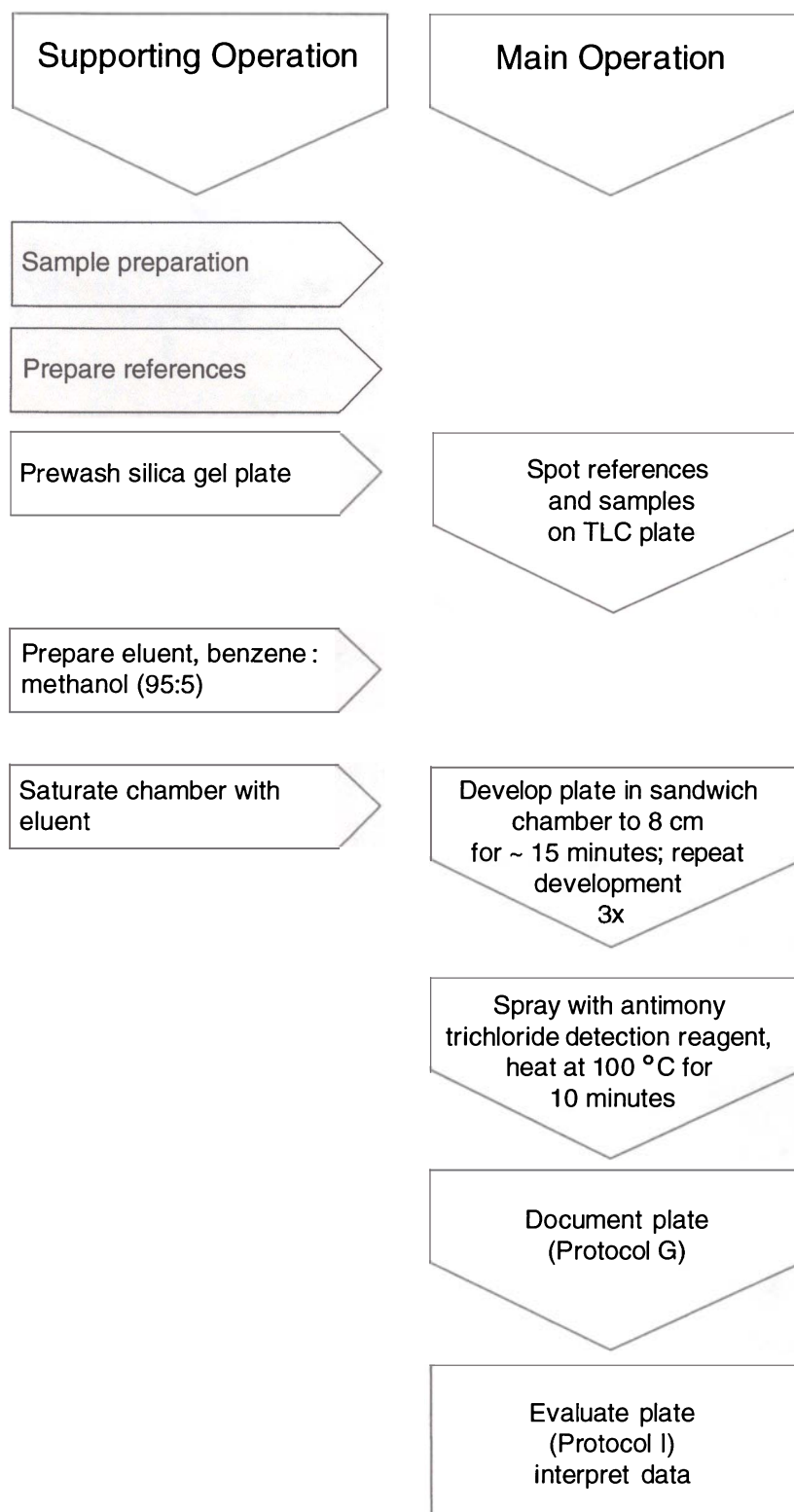
| Supporting Operation | Main Operation |
|---|---|

Sample preparation

Prepare references

Prewash silica gel plate

Spot references
and samples
on TLC plate

Prepare eluent, benzene :
methanol (95:5)

Saturate chamber with
eluent

Develop plate in sandwich
chamber to 8 cm
for ~ 15 minutes; repeat
development
3x

Spray with antimony
trichloride detection reagent,
heat at 100 °C for
10 minutes

Document plate
(Protocol G)

Evaluate plate
(Protocol I)
interpret data

## 1. Equipment and Supplies

The following equipment is needed for a single development TLC analysis using a sandwich chamber:

1) Amber glass storage bottles (250 ml)
2) Capillary pipettes (1.0 and 0.2 µl size)
3) Hot plate
4) Thermometer
5) Cylindrical TLC chamber and lid
6) Sandwich chamber
7) Glass vials with Teflon septum caps (4 ml)
8) 250-µl vial inserts and compression springs
9) Graduated cylinder (100 ml)
10) Oven
11) Reagent sprayer
12) Ruler (inch and metric)
13) Spray box
14) Spray stand

## 2. Chemicals and Materials

1) Ethyl acetate
2) Eluent components
   *Benzene*
   *Methanol*
3) Merck HPTLC silica gel plate, 10 x 10 cm
4) Methanol
5) Antimony trichloride detection reagent (Sigma Chemical Co.)
   **(Caution: toxic detection reagent, handle with care)**
6) Resin reference materials in ethyl acetate (3 mg/ml)

| | |
|---|---|
| *Amber* | *Gamboge* |
| *Benzoin* | *Manila copal* |
| *Colophony* | *Mastic* |
| *Congo copal* | *Myrrh* |
| *Dammar* | *Shellac* |
| *Dragon's blood* | *Sandarac* |
| *Elemi* | |

## 3. Samples

Samples can be taken from facsimile objects or unknowns. The sample should be approximately 500 µg in weight and contain only the paint layer or material of interest. The paint layer or material being investigated should be separated from all other layers, such as the ground, varnish layers, or support. The sample is dissolved in ethyl acetate (some of the sample may not totally dissolve in the solvent). To do this, place the sample in a 250-µl insert vial and add

100 µl of ethyl acetate. Place the insert vial in a compression ring and put
the entire unit in a 4-ml glass vial. Then seal the vial with the Teflon septum cap.



Insert vial      Compression spring      Complete assembly inside 4-ml vial
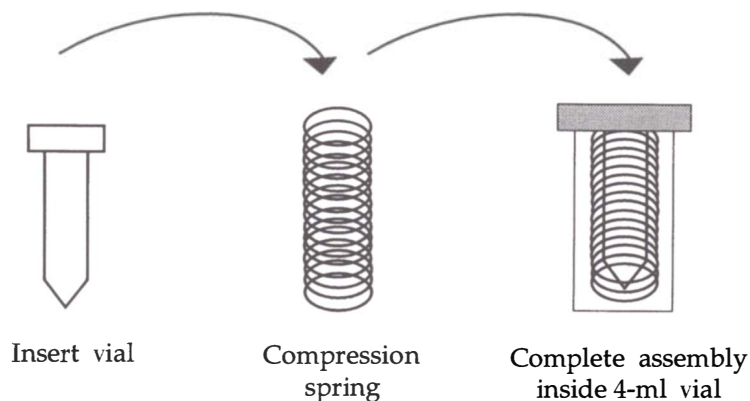
Reference solutions of binding media are made from amber, benzoin, colophony,
Congo copal, dammar, dragon's blood, elemi, gamboge, Manila copal, mastic,
myrrh, shellac, and sandarac. All reference solutions are made in a concentration
of 3 mg/ml. They are prepared by weighing 6 mg of resin into a 4-ml glass
vial and adding 2 ml of ethyl acetate. This vial is then sealed with the
Teflon septum cap. These solutions are prepared at least 24 hours before
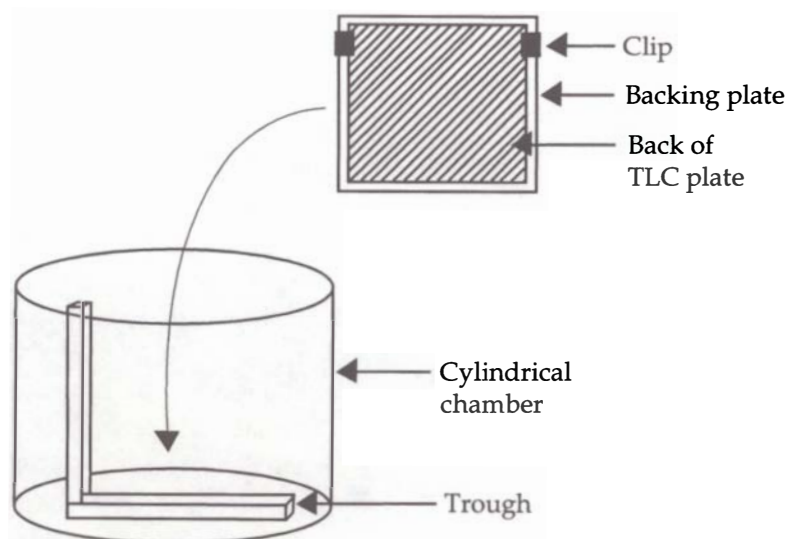analysis and are stable for months at room temperature.

## 4. Preparation Procedures

Preparation for TLC analysis includes prewashing and activating the TLC plate and
making fresh eluent systems. The following preparation procedures are started
24 hours prior to analysis:

1) **Prepare silica TLC plates**

   The silica gel plate is washed with methanol, then heated before use. This
   procedure takes about 2 hours. The plate can be washed in a sandwich
   chamber or a conventional chamber. The procedure described here uses a
   sandwich chamber.

   Place 5 ml of methanol in the trough of a TLC sandwich chamber. Position
   the silica gel plate so that the stationary phase faces the backing plate and
   adjust the spacing with the spacing guide. Attach the silica plate to the
   backing plate with two clips. Make sure that the clips are attached near the
   top of the plate. Insert the assembly vertically into the trough containing
   the methanol. Place all parts inside a cylindrical TLC chamber and cover with
   the lid. Allow the methanol to rise to the top of the silica gel plate. This
   process usually takes 20 minutes. Remove the plate from the chamber and
   dry it in a fume hood. Place the plate in an oven at 100 °C for 1 hour to
   activate the silica gel. Cool and store the clean, activated silica plate in a
   desiccator containing silica gel. The plate is ready for use after it cools
   (usually 30 minutes after it is removed from the oven).

2) **Prepare eluent**

   Prepare 60 ml of the eluent fresh daily for an analysis. Mix benzene and methanol in a 95:5 ratio. Seal the solution in an amber bottle to maintain freshness.

3) **Prepare cylindrical chamber**

   The TLC chamber must be saturated with 10 ml of the eluent at least 30 minutes before analysis. Place the trough of the sandwich chamber inside the cylindrical chamber. Pour 10 ml of the eluent solution into the cylindrical chamber and cover it with a lid.
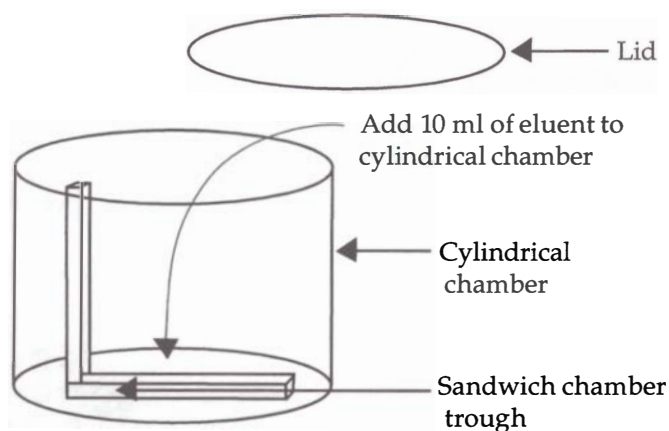


## 5. TLC Analysis Procedures

To analyze resins by TLC, the ethyl acetate extracts of samples are spotted in individual lanes at the baseline of a prewashed, activated HPTLC silica gel plate. The plate is attached to the backing plate of a sandwich chamber and inserted into the small trough containing the eluent (benzene : methanol, 95:5). The development of the plate takes place inside a cylindrical TLC chamber to minimize the effects of air currents on the movement of the solvent front. This also mini-

mizes the premature evaporation of the solvent system. The development of the plate is complete when the eluent front reaches a distance of 8 cm from the baseline. Depending on the nature of the unknown resin and the need for resolution, the plate may be developed two or three times. The plate is removed from the sandwich chamber and dried in a fume hood. The eluent is replenished if necessary and the plate is redeveloped. The plate is dried, then sprayed with an antimony trichloride detection reagent. Patterns of fluorescent spots are visible for each sample when the plate is viewed under ultraviolet light. The plate may be photographed for documentation immediately.

The following twelve steps describe the procedure for analysis:

1) **Draw the baseline**

Using a ruler and pencil, lightly draw a line 1 cm from the bottom edge of the plate. Very lightly mark the lanes with short tick marks at intervals of 0.5 cm along this baseline, for a total of 19 lanes. In the upper left corner, number the plate with a reference number, used to relate the TLC to information in the research notes. Beside the number, place the date and the operator's initials. Place a mark 8 cm from the baseline as a reference to help determine the completion of the development. If the mark is scored heavily through the silica gel, it will be visible on both sides of the plate.
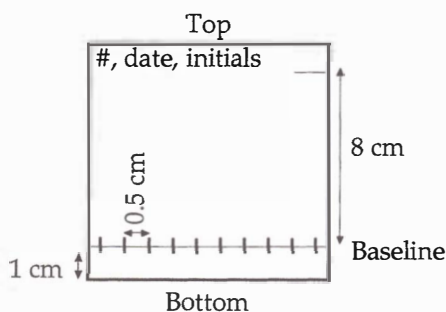


2) **Apply the reference solutions to the plate**

All solutions are applied following the spotting procedures noted in **Protocol H.** Approximately 10 minutes before analysis, gently heat the samples to 40 °C on a hot plate. Apply 0.5 µl of each reference solution to a tick mark on the origin of a lane using a capillary pipette. The total volume may be applied in a series of smaller volumes to minimize the diameter of the spot. An air gun may be used to rapidly evaporate the carrier solvent between applications. *Take care not to get the air gun too close to the pipette, as the sample will evaporate.*

3) **Apply unknown sample solutions**

Heat the samples to 40 °C 10 minutes before application. If possible, apply each unknown sample in two different volumes. For example, in one lane apply 0.5 µl of the unknown sample, and in a second lane apply 1 µl of the same solution. (The unknown sample may or may not be very concentrated, and this procedure minimizes the possibility of overloading the plate.)

4) **Develop the TLC plate**

Once the plate is spotted, develop immediately or store in a desiccator. Open

the lid of the cylindrical chamber slightly and quickly place 5 ml of eluent solution in the trough of a TLC sandwich chamber, located inside the cylindrical chamber. Position the silica gel plate so that the stationary phase faces the backing plate and adjust spacing between the edge of the plate and the rim of the backing plate with a spacing guide. Attach the silica plate to the backing plate with two clips. Make sure that the clips are attached near the top of the plate, next to the spacers. Quickly open the chamber and set the bottom edge of this assembly into the trough containing the eluent. Replace the lid of the chamber. *Do not leave the chamber open for any length of time, as the vapor phase equilibrium will be lost.*
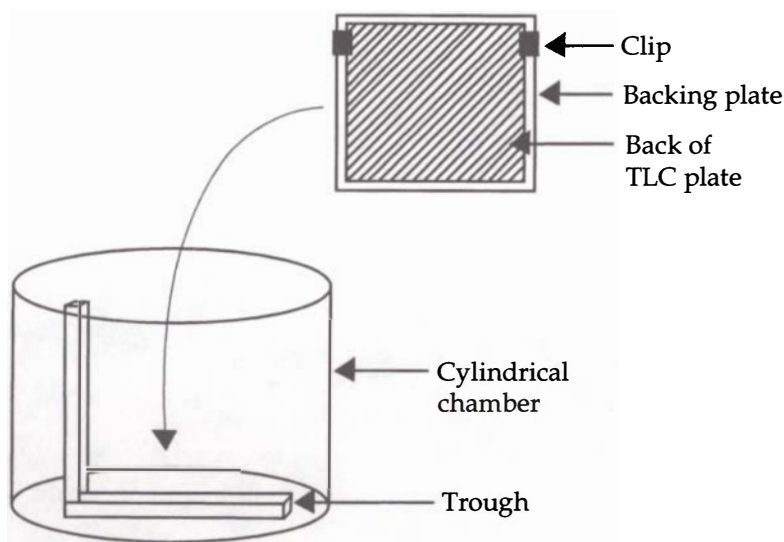


5) **Completion of development**
   Develop the plate until the solvent front reaches the 8 cm mark on the plate. Development usually takes about 15 minutes.

6) **Dry the plate**
   Remove the plate from the chamber and let it dry for about 30 minutes at room temperature in the fume hood.

7) **Redevelop the plate**
   Reattach the TLC plate to the backing plate of the sandwich chamber, as before. Check the spacing of the plate with the spacing guide. Replenish the eluent in the trough if necessary. Place the bottom assembly in the trough and cover the cylindrical chamber, as before.

8) **Completion of second development**
   Develop the plate to the mark of the first development, which usually takes about 30 minutes. The plate may be redeveloped a third time if higher resolution of the chromatographic spots is desired.

9) **Dry the plate**
   Remove the plate from the chamber and let it dry for about 30 minutes at room temperature in the fume hood.
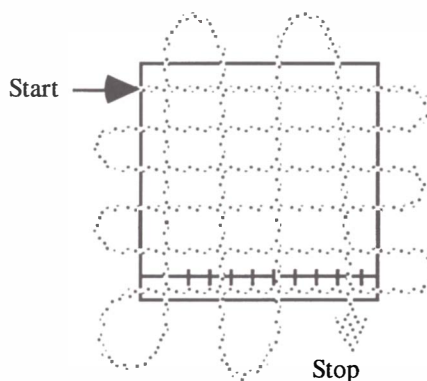
10) **Prepare to spray plate**
    **Note: Spraying of an antimony trichloride reagent should always be performed under a well-ventilated fume hood or some other device to**

**ensure effective removal of the reagent cloud and solvent vapors, which are corrosive and toxic. Protective glasses, laboratory gloves, and a respirator should always be worn during spraying.** Set the plate on a clean, dry spray stand inside a spray box. Fill the reagent sprayer with 15–20 ml of antimony trichloride detection reagent.

11) **Spray plate with antimony trichloride**

Hold the reagent sprayer 8–10 cm from the surface of the TLC plate and spray the plate slowly back and forth, then up and down, until the plate is evenly covered. This is usually when the plate just begins to turn transparent.



12) **Heat plate**

Dry the plate for 15–20 minutes in the fume hood, then place it in a preheated oven at 100 °C for 10 minutes.

## 6. Data Analysis Procedures

After the separation zones are visualized by reaction with the detection reagent, the plate is documented (see **Protocol G**) and evaluated (see **Protocol I**). Documentation includes two procedures. The first is written documentation that includes annotation in a bound research notebook of all methods and procedures used. The second is visual documentation, which includes making a photocopy of the TLC plate (to be placed in the notebook) and photographing or digitally scanning the plate. Evaluation of the plate can include qualitative or semiquantitative techniques. The migration distances, color, and intensity of the separation spots are noted. The $R_f$ value for each spot is calculated.

## 7. Interpretation

The chromatograms produced by these procedures contain a large number of spots that fluoresce in a variety of colors. The chromatographic pattern of the unknown materials is compared to those of the reference materials run on the same plate. Both the locations and the colors of the spots are significant to the interpretation of the data. Identification of the unknown sample can be made if the chromatographic pattern of both the location and the colors closely matches that of a reference sample.

Protocol E

# Written Documentation of the TLC Plate

**Summary**

Guidelines for written documentation of a TLC plate

Supplies:   Bound research notebook
            Waterproof black ink pen
            Archival adhesive
Time:       Approximately 15 minutes per plate

**Scope**  It is good laboratory practice to maintain complete written and photographic documentation of each TLC plate. One rule of thumb is that the experiment should be completely reproducible from the written notes. This protocol provides a template for creating an accurate description of the methods and procedures used in a TLC experiment.

The chromatographic data needed for reporting a TLC experiment include information on the chamber, plate, eluent, application and location of the samples, and detection reagents. This information is logged in a bound research notebook as the experiment proceeds. Upon completion of the experiment, the TLC plate is evaluated, and $R_f$ values of the separation zones are recorded. The photocopy of the TLC plate is made and placed in the research notebook using an archival adhesive.

### 1. Documentation of a TLC Plate

The written documentation of a TLC plate is an important part of the overall research process. Written documentation is kept for every TLC experiment attempted, whether the results are positive, negative, or inconclusive. The data provide a means of examining the work, and aid in the design of new experiments.

For example, an unknown is analyzed for the identification of proteins by TLC. It is hydrolyzed and spotted at two volumes, 1.0 and 0.2 µl, on a cellulose plate. Upon detection of the plate, very faint spots are seen for the 1.0-µl application and no spots are seen for the 0.2-µl application of the unknown. By looking at the written notes, it becomes obvious that the concentration of the unknown sample is too low. The sample can be concentrated and reanalyzed using the same method. It is also useful to make a photocopy of the TLC plate and keep it with the written notes.

### 2. Procedures

All experimental information is entered into a bound research notebook with a black waterproof ink pen. Any mistakes in the notes are marked out with a single stroke through the error. Corrections are written beside or above the mistake. Notes should be written neatly so that others can read them. It is suggested that instructions be written in first person, active voice. For example: "I placed the TLC plate into the chamber at 3:00 P.M."

1) **Copy template into research notebook prior to analysis**
   In order to note all the important details of the experiment while it is in progress, it is helpful to copy the following template into the research notebook prior to analysis.
2) **Record the following information as the analysis proceeds:**
   a. Date
   b. Name of technician
   c. Type of TLC analysis
   d. Purpose of analysis (1–2 sentences)
   e. Type of chamber and approximate dimensions
   f. Type of development (i.e., sandwich chamber or conventional; single or multiple)
   g. Eluent system (with manufacturer and lot of solvents used)
   h. Type of TLC plate (with manufacturer and lot used)
   i. Preparation of TLC plate (i.e., prewashed and activated or as received)
   j. Start time of analysis
   k. Stop time of analysis
   l. Total time
   m. Distance traveled by eluent
   n. Detection reagent (with manufacturer and lot used, and date prepared)
3) **Prepare a table and record the following:**
   a. Lane number
   b. Substance applied
   c. Quantity of sample applied

     d. Date solution was prepared (and notebook page number)

     e. Number of separation zones

       i. distances

       ii. $R_f$ values

       iii. notes

4) **Make a photocopy of the TLC plate**

After the detection and evaluation of the TLC plate, make a photocopy of the plate and adhere it to the notebook with an archival adhesive. **Note: Do not use tape**. An acid-free white glue is recommended for attaching notes, photocopies of plates, etc., to the notebook.

**Template for Written Documentation of a TLC Plate**

| Date: | Name: |
|---|---|
| Type of analysis: | |
| Purpose: | |

| | |
|---|---|
| Chamber: | Type of development: |
| Eluent system: | |
| TLC plate: | Preparation of plate: |
| Start time: | Stop time: |
| Total time: | Distance traveled: |
| Detection reagent: | |

| Lane | Substance | Quantity | Distance/$R_f$ values | Notes |
|---|---|---|---|---|
| 1. | | | | |
| 2. | | | | |
| 3. | | | | |
| 4. | | | | |
| 5. | | | | |
| 6. | | | | |
| 7. | | | | |
| 8. | | | | |
| 9. | | | | |
| 10. | | | | |
| 11. | | | | |
| 12. | | | | |
| 13. | | | | |
| 14. | | | | |
| 15. | | | | |
| 16. | | | | |
| 17. | | | | |
| 18. | | | | |
| 19. | | | | |

Protocol F

## Photodocumentation of the TLC Plate Using Visible Light

**Summary**

Guidelines for photographic documentation of a TLC plate

| | |
|---|---|
| Equipment: | 35-mm camera |
| | Copy stand |
| | Light box (similar to those used to view slides) |
| | Two tungsten lamps |
| | Light meter (optional) |
| Film: | Black-and-white film: 35-mm Kodak TMAX 100 |
| | Color slide film: 35-mm Kodak Ektachrome Professional 160T (tungsten balanced) |
| Time: | Approximately 30 minutes per plate (less in a large series) |

**Scope**  Photography is used to quickly and accurately record the TLC results under either normal or ultraviolet light. This protocol describes the procedures used to photograph a TLC plate under normal light. For photodocumentation of TLC plates under ultraviolet light, see **Protocol G**.

Photography is the preferred method for permanently recording the results of a TLC experiment. TLC separation zones can be detected with reagents that create visible spots, such as those seen for amino acids when detected with ninhydrin. Such a TLC plate is photographed using tungsten photoflood lamps (balanced 3200 °K) for illumination. The TLC plate is photographed with black-and-white print film and color slide film under normal illumination using a 35-mm camera on a copy stand.

**Scheme**

**Documentation of Thin-Layer Chromatography by Normal Photography**

| Supporting Operation | Main Operation |
|---|---|

Load film

Mount filters on camera

Set up copy stand and mount camera

Position TLC plate on the light box

Set up the tungsten lamps

Meter lighting—to determine approximate exposure

Photograph and bracket shots

Document and log each shot

Rewind film; send for development

Label and store slides and prints

## 1. Equipment and Supplies

For photographic documentation of a TLC plate by normal photography, the equipment and supplies needed are:

1) 35-mm camera with lens
2) Copy stand
3) Light box (like those used for viewing slides)
4) Two tungsten photoflood lamps (balanced 3200 °K)
5) Light meter
6) Black-and-white film: 35 mm
   *Kodak TMAX 100*
7) Color slide film: 35 mm
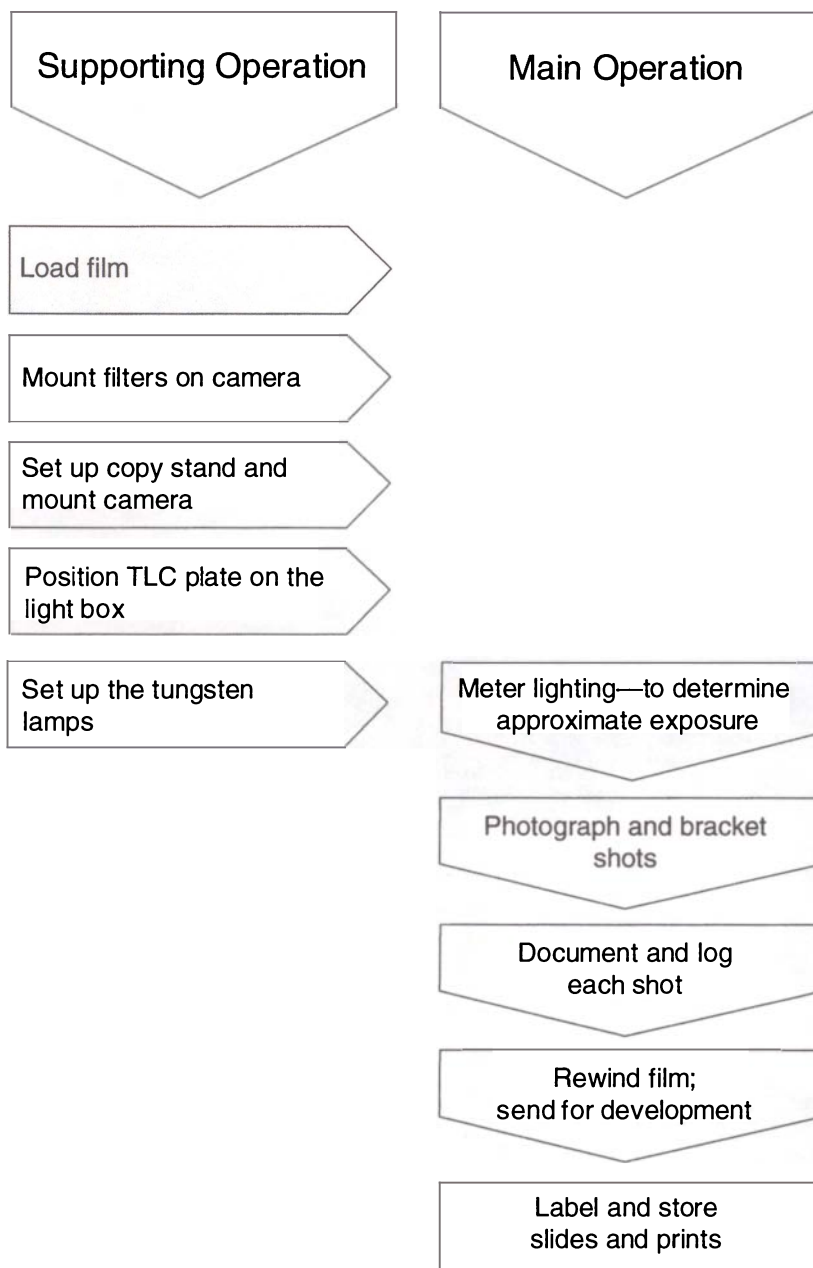   *Kodak Ektachrome Professional 160T (tungsten balanced)*

## 2. Documentation of TLC Plates

Plates are selected for photographic documentation. Generally, only positive results from successful TLC separations are photographed, depending on the amount of time and supplies available.

## 3. Preparation Procedures

1) **Choose and load film**

   A 35-mm camera is used for the photodocumentation of the TLC plate. Generally, both a black-and-white print and a color slide are made of the TLC plate. If two camera bodies are available, one can be loaded with the black-and-white film, and the other with the slide film. The camera bodies can be interchanged, using a single lens, during the documentation of the plate. Relatively slow-speed films are used for the documentation of the visible separation zones found on the TLC plate. Films used for this documentation include the Kodak TMAX 100 black-and-white print film and the Kodak Ektachrome Professional 160T color slide film. These films are used because of the fine grain and excellent reproduction and enlargement capabilities. If lighting conditions are limited—that is, if the exposures are too long—faster films such as Scotch 640T tungsten-balanced slide film or Kodak TMAX 400 black-and-white film may be effective.

2) **Set up copy stand and mount camera**

   The copy stand is set up in a convenient location that is away from other light sources, such as fluorescent lighting *(this can cause a green cast to your color slides)*. A standard 50-mm lens (or a 60-mm macro lens, depending on the size of the plate) is attached to the camera body, which is mounted in turn on the arm of the copy stand.

3) **Set up the light box and position the TLC plate**

   A light box is placed at the center of the copy stand and plugged into an electrical socket. The chromatographic plate is placed on the light box and centered in the viewfinder of the camera. The light box is switched to the "on" position, and the TLC plate is viewed under transmitted light.

4) **Set up the tungsten lamps**

   The TLC plate can be photographed using only the transmitted light from

the light box, or the light can be supplemented with two photographic tungsten lamps. Often, the plate will first be photographed using transmitted light, then both transmitted and reflected light. One lamp is placed at 45° on each side of the TLC plate, and positioned so that the TLC plate is evenly illuminated.
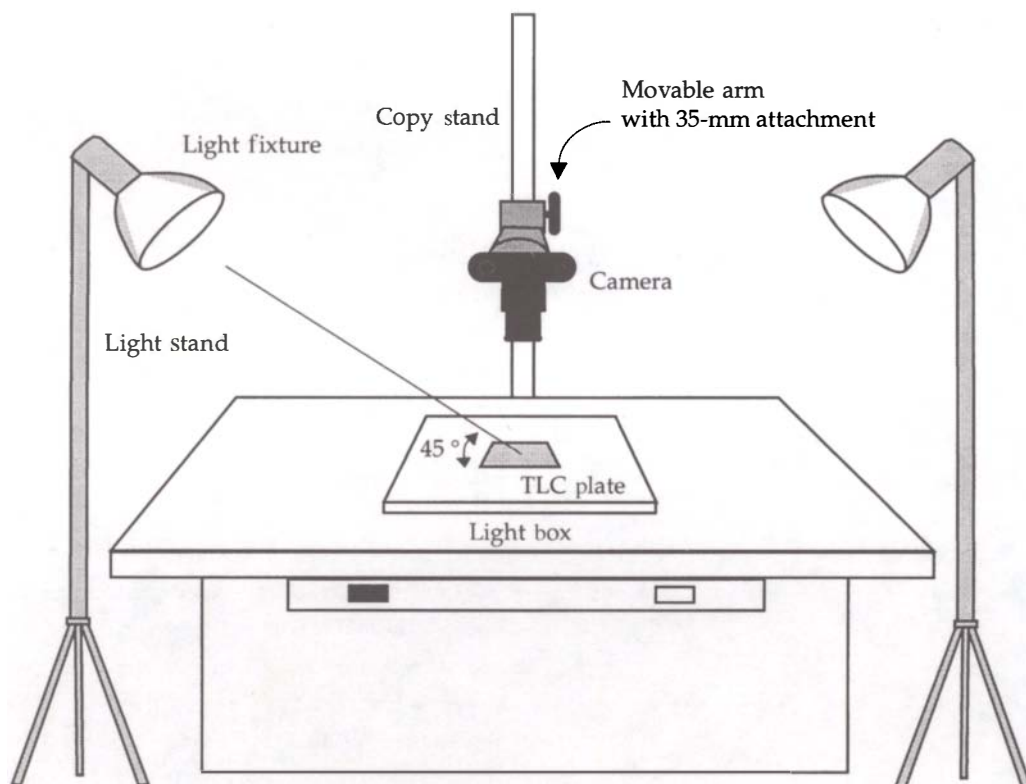


### 4. Photodocumentation Procedures

1) **Meter lighting to determine exposure**
   The exposure is determined by TTL (through the lens) metering, using the built-in light meter of the camera. The reflected light from the TLC plate can alternatively be metered with a handheld light meter. It is recommended that the f-stop be set at f 8.0—which provides a good depth of field—and that the exposure time be adjusted until the proper exposure is reached.

2) **Photograph and bracket shots**
   Make sure that the camera ASA is set to the appropriate ASA for the film being used and that the f-stop is set at f 8.0. Photograph the plate with the exposure time set to the metered reading. Bracket the exposure by changing the f-stop up one stop, then down one stop, from the metered exposure. This provides a range of exposures and betters the chance of taking a good negative or slide. It is easier to capture a good image of the plate within 24 hours of detection than to rephotograph the plate at a later time. *(An*

*exception to this rule is the photodocumentation of proteins detected by ninhydrin. In this case, the separation zones are more easily seen after 24 hours. TLC plates for protein analysis should be photographed between 24 and 48 hours after detection.)*

3) **Document and log each shot**
It is advisable to keep a written document of the photographs. This information can be kept in a laboratory notebook, in a ring binder, or on a computer database. The information is valuable in that it provides documentation on the appropriate exposure for the photograph. If the photographs do not come out satisfactorily, the information can be useful in making exposure corrections. It also allows for tracking of photographs, particularly for multiple originals that may be similar. Most important, should the photograph need to be duplicated at a later date, all necessary information on the exposure has been retained. A sample log sheet is attached (Attachment A).

4) **Rewind film and send for development**
After all exposures are made, rewind and remove the film from the camera. Send the film to a reliable photo processing laboratory. Ask the lab to number the slides, as this helps correlate each shot with written documentation. Black-and-white film is processed and printed as a proof sheet. Individual photographs can be chosen for printing from the proof sheet or the slide.

5) **Label and store slides and prints**
Labeling of slides is strongly encouraged. This is particularly useful when slides are borrowed by colleagues for presentations, so that the slides can be easily refiled. Proof sheets should be placed in 8 x 10" polyethylene sheet protectors, and negatives can be housed in negative holders. The proof sheets and the negatives can then be stored in D-ring binders.
Slide labels can be handwritten, typed, or generated by computer. The label should contain at least: *Name, Date,* and *Subject.* Other useful information can be added to the label, as well. A sample label may look like this:

| Date | Roll No. | | Photo No. |
|------|----------|-----|-----------|
| Subject | | | |
| Camera | Lens | f-stop | Shutter speed |
| Film | | Photographer | |

PROLaser labels are designed to fit slide mounts and can be printed on laser printers. They can be obtained through photographic suppliers or ordered directly from the manufacturer. For direct orders, write to: Slide Scribe, 752 Washington Avenue So., Minneapolis, MN 55439.

*Helpful Hint: A dot can be placed in the upper right corner of each label and used to orient slides for projection. First, the slide is held with the image upside down. Next, the label is attached to the front face of the slide, on the top border. The dot can be used to determine the proper orientation of the*

*slide. When all slides are properly placed in the slide carousel, the dot will be seen on the outer edge of the slide.*

Always use polyethylene holders for photographic images, as nonarchival materials will damage the image. For valuable slides, make duplicates and store one copy in a separate place.

Attachment A

**Photodocumentation Log Sheet**

Film roll no. _____

Name _____          Date _____

Subject  _____

_____

_____

Lights _____          Correction filters _____

Camera _____          Lens _____

Meter _____          Polaroid _____

Diagram of equipment setup

Name _____

Film roll no. _____

| # | Sample | Camera distance | f-stop | Shutter | Filter | Description | Comments |
|---|--------|-----------------|--------|---------|--------|-------------|----------|
| 1 | | | | | | | |
| 2 | | | | | | | |
| 3 | | | | | | | |
| 4 | | | | | | | |
| 5 | | | | | | | |
| 6 | | | | | | | |
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| 32 | | | | | | | |
| 33 | | | | | | | |
| 34 | | | | | | | |
| 35 | | | | | | | |
| 36 | | | | | | | |

Protocol G

## Photodocumentation of the TLC Plate Using Ultraviolet Light

**Summary**

Guidelines for photographic documentation of a TLC plate by ultraviolet fluorescence photography

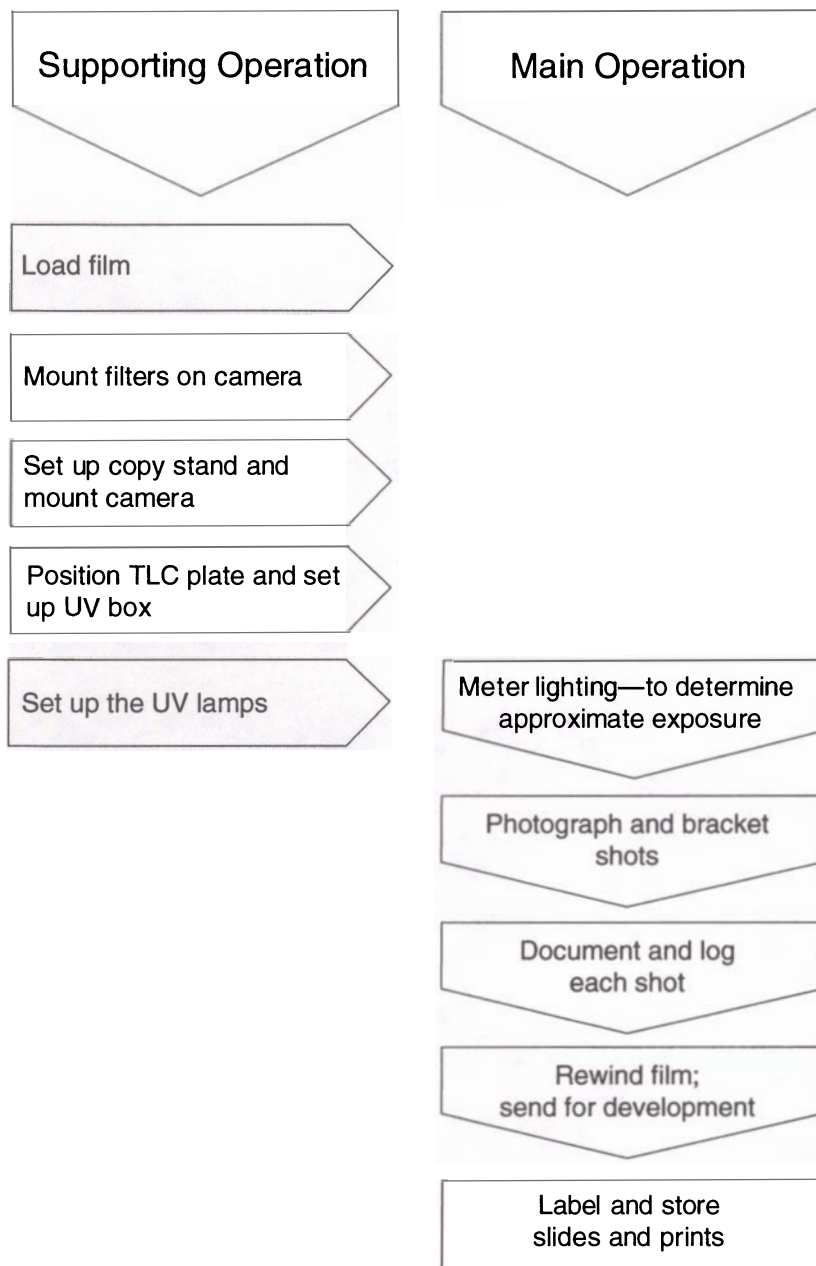| Equipment: | 35-mm camera |
| | Copy stand |
| | Ultraviolet light box (see Attachment A) |
| | Two handheld ultraviolet lamps |
| | Light meter (optional) |
| Filters: | Kodak Wratten gel filters (2E, 10R, 10M, 20Y) |
| Film: | Black-and-white film: 35-mm Kodak TMAX 400 |
| | Color slide film: 35-mm Fujichrome Professional 400 |
| Time: | Approximately 30 minutes per plate (less in a large series) |

**Scope**

Photography is used to quickly and accurately record the TLC results under either visible or ultraviolet light. This protocol describes the procedures used to photograph a TLC plate under ultraviolet light. For photodocumentation of TLC plates under visible light, see **Protocol F**.

Photography is the preferred method for permanently recording the results of a TLC experiment. TLC plates detected with ultraviolet fluorescent reagents, such as those used for the analysis of carbohydrates, waxes, and resins, are photographed under the illumination of ultraviolet lamps. The separation zones of the chromatogram are seen as visible fluorescent spots. The TLC plate is photographed with black-and-white print film and color slide film under ultraviolet illumination, using a 35-mm camera on a copy stand.

## Scheme

## Documentation of Thin-Layer Chromatography by Ultraviolet Fluorescence Photography

| Supporting Operation | Main Operation |
|---|---|

**Supporting Operation:**

Load film

Mount filters on camera

Set up copy stand and mount camera

Position TLC plate and set up UV box

Set up the UV lamps

**Main Operation:**

Meter lighting—to determine approximate exposure

Photograph and bracket shots

Document and log each shot

Rewind film; send for development

Label and store slides and prints

## 1. Equipment and Supplies

For photographic documentation of a TLC plate by ultraviolet fluorescence photography, the equipment and supplies needed are:

1) 35-mm camera
2) Copy stand
3) UV light box
4) Two handheld UV lamps
5) Light meter
6) Kodak Wratten gel filters and holder
    *#2E*
    *#10R*
    *#10M*
    *#20Y*
7) Black-and-white film: 35 mm
    *Kodak TMAX 400*
8) Color slide film: 35 mm
    *Fujichrome Professional 400*

## 2. Documentation of TLC Plates

Plates are selected for photographic documentation. Generally, only positive results are photographed, depending on the amount of time and supplies available.

To photograph fluorescence, the room must be in total darkness; all visible light (except that originating from the plate) must be excluded. A photographic darkroom is the ideal location to photograph these plates. If one is not available, a simple UV light box can be constructed that can be used in conjunction with a darkened room. Due to the low level of visible light being recorded, fast films are exposed and "push-processed" to achieve satisfactory images.

## 3. Preparation Procedures

1) **Choose and load film**
    A 35-mm camera is used for the photodocumentation of the TLC plate. Generally, both a black-and-white print and a color slide are made of the TLC plate. If two camera bodies are available, one can be loaded with the black-and-white film, and the other with the slide film. The camera bodies can be interchanged during the documentation of the plate.
    High-speed films are needed for the documentation of the visible fluorescence resulting from the illumination of the plate with ultraviolet light. Films used for ultraviolet fluorescence photography include the Kodak TMAX 400 black-and-white print film and the Fujichrome 400 color slide film. These films are exposed at an 800 ASA setting and "push-processed" one f-stop at a professional film processing lab. The film is loaded into the camera

according to the procedures specified in the camera manual. Set the camera ASA to 800, and set the f-stop to f 8.0.

2) **Choose and mount filters on camera**

Gel filters are used with ultraviolet fluorescence photography to eliminate reflected ultraviolet radiation and, in the case of color slide film, to correct the color balance of the film so that the resulting slide will accurately reproduce the color of the fluorescent spots seen. Gel filters are rather fragile and easily deteriorate; they should be handled carefully.

The Kodak Wratten gel filter 2E is used for black-and-white prints. For color slides, the Kodak Wratten gel color correction filters designated 10R, 10M, and 20Y are used in addition to the 2E filter. The order in which the filters are loaded into the holder is important. The 2E filter, which blocks ultraviolet radiation, should be placed closest to the TLC plate being photo-graphed. The color correction filters are placed between the 2E filter and the lens.

3) **Set up copy stand and mount camera**

The copy stand is set up in a room that can be made completely dark. Although a photographic darkroom is ideal, a closet or other windowless room can be used. A standard 50-mm lens (or a 60-mm macro lens, depending on the size of the plate) is attached to the camera body, which is mounted in turn on the arm of the copy stand.

4) **Position TLC plate and set up the UV light box**

(See Attachment A of this protocol for instructions on the manufacture of an inexpensive UV box.) Place the TLC plate on the base of the copy stand, and center the plate while looking through the viewfinder of the camera. Place the UV light box over the TLC plate and position it so that the opening is aligned with the lens of the camera. Move the arm of the copy stand so that the vertical position of the lens is at or near the opening of the box.

5) **Set up UV lamps**

Two laboratory UV lamps are used to illuminate the TLC plate. One lamp is placed on each side of the box. Each lamp is positioned so that the lamp area is flush with the window or opening of the box.

## 4. Photodocumentation Procedures

1) **Determine exposure**

For ultraviolet fluorescence, it is possible to approximate the exposure from a light meter reading with a handheld light meter (see step 2 if a meter is not available). The meter must be capable of calculating long exposures. The meter is used to measure the reflected light coming from the plate. First, turn on both UV lamps (in the shortwave mode). Look through the camera to see that the TLC plate is centered in the viewer. Check to see that the UV box is not blocking any part of the image. Move the copy stand arm, and back the camera away from the opening of the box. Set the handheld light meter to an f-stop of f 8.0 and the ASA/ISO to 800. Place the light meter directly over the opening and meter the exposure time. Since the colored filters that are placed in front of the lens absorb some of the light that would

normally reach the film, the exposure must be adjusted. Following is a list of the filter factors for each of the filters used:

| Filter | Exposure increase in f-stops |
| --- | --- |
| 2E | N/A |
| 10R | 1/3 |
| 10M | 1/3 |
| 20Y | 1/3 |

The amount of correction needed is the sum of the exposure increase for the filters that are used. For example, for a color slide, all four filters are used, so the exposure is increased by one f-stop.

2) **Photograph and bracket shots**

Make sure that the camera ASA is set to 800 and the f-stop is set to f 8.0. Photograph the plate with the exposure time set to the metered reading. Remember that the gel filters affect the exposure by lowering the amount of light reaching the film. The film will be underexposed at the metered reading, but it is a good starting point. For example, if the metered exposure indicates an exposure time of 4 seconds, then exposures of 4, 8, and 16 seconds are likely to produce at least one negative that is properly exposed. If a light meter is not available, start with an exposure of 1 second and double the exposure time with each shot to 32 seconds. For example, take a photograph with the exposure time set to 1 second. Then set the camera exposure to the bulb position and take photographs at 2, 4, 8, 16, and 32 seconds. Timing can be done with a darkroom clock or by simply counting the time.

3) **Document and log each shot**

It is advisable to keep a written document of the photographs. This information can be kept in a laboratory notebook, in a ring binder, or on a computer database. The information is valuable in that it provides documentation on the appropriate exposure for the photograph. If the photographs do not come out satisfactorily, the information can be useful in making exposure corrections. It also allows for tracking of photographs, particularly for multiple originals that may be similar. Most important, should the photograph need to be duplicated at a later date, all necessary information on the exposure has been retained. A sample log sheet is attached (Attachment B).

4) **Rewind film and send for development**

After all exposures are made, rewind and remove the film from the camera. Send the film to a reliable photo processing laboratory. Indicate to the lab that the film has been exposed at 800 ASA and should be "push-processed." Also ask the lab to number the slides, as this helps correlate each shot with written documentation. Black-and-white film is processed and printed as a proof sheet. Individual photographs can be chosen for printing from the proof sheet or the slide.

5) **Label and store slides and prints**

Labeling of slides is strongly encouraged. This is particularly useful when slides are borrowed by colleagues for presentations, so that the slides can be easily refiled. Proof sheets should be placed in 8 x 10" polyethylene sheet protectors, and negatives can be housed in negative holders. The proof sheets and the negatives can then be stored in D-ring binders.

Slide labels can be handwritten, typed, or generated by computer. The label should contain at least: *Name, Date,* and *Subject.* Other useful information can be added to the label, as well. A sample label may look like this:

| Date | Roll No. | | Photo No. |
| --- | --- | --- | --- |
| Subject | | | |
| Camera | Lens | f-stop | Shutter speed |
| Film | | Photographer | |

PROLaser labels are designed to fit slide mounts and can be printed on laser printers. They can be obtained through photographic suppliers or ordered directly from the manufacturer. For direct orders, write to: Slide Scribe, 752 Washington Avenue So., Minneapolis, MN 55439.

*Helpful Hint: A dot can be placed in the upper right corner of each label and used to orient slides for projection. First, the slide is held with the image upside down. Next, the label is attached to the front face of the slide, on the top border. The dot can be used to determine the proper orientation of the slide. When all slides are properly placed in the slide carousel, the dot will be seen on the outer edge of the slide.*

Always use polyethylene holders for photographic images, as nonarchival materials will damage the image. For valuable slides, make duplicates and store one copy in a separate place.

Attachment A
**Instructions for the Manufacture of an Inexpensive UV Box**

Cardboard boxes can be used to make an inexpensive chamber for ultraviolet fluorescence photography. First, the top flaps of the box are removed with a utility knife. Then, two right triangles are cut from one side of the box.
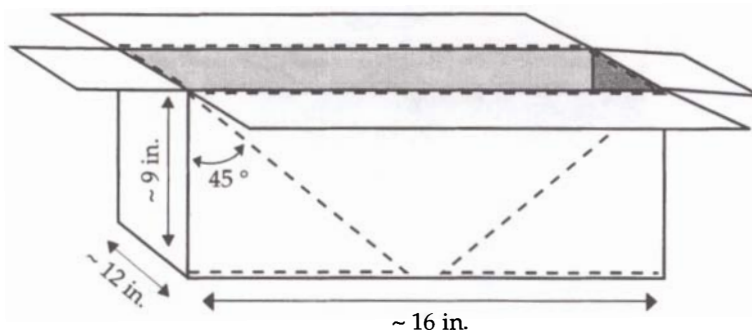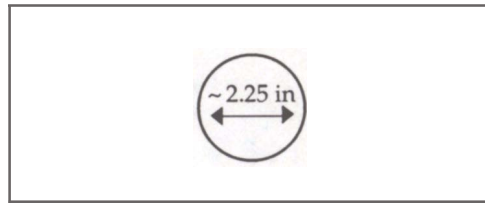


1. Cut along dotted lines and remove the top flaps. Be sure to cut evenly so that the box sits flat.

2. Cut two right triangles into one side of the box.

Next, a round opening 2.25 inches in size is cut in the center of the bottom of the box.
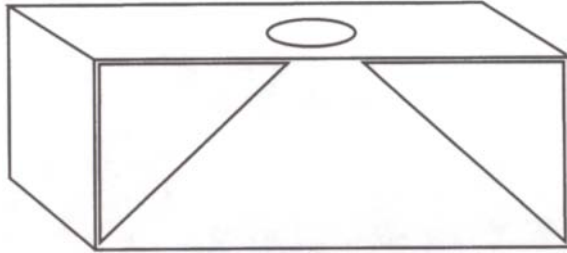
Side view

3. Cut an opening into the bottom of the box so that a camera lens will fit snugly.

Overhead view

Two inserts are made from extra cardboard or a second box. Each cardboard insert is cut to a length that is 1 inch longer than the width of the UV box (about 13 inches). Cut the width of the insert so that two 1-inch external tabs remain. Two tabs are also cut on one end of the insert, and a rectangle is cut from the center. Cut along the dotted lines as indicated in the diagram below.

~ 1 in.

~ 6.5 in.

~ 3 in.
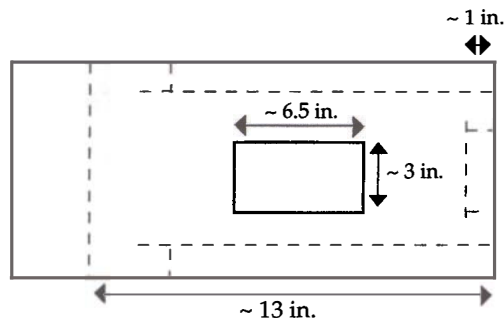
~ 13 in.

Cutting diagram
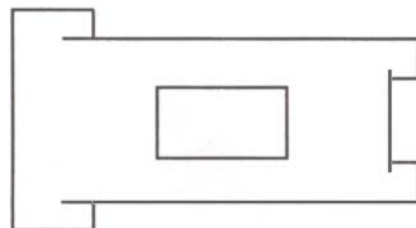
4. Use a flat piece of cardboard cut from a second box to manufacture an insert.

5. Cut the length of the cardboard insert to equal the width of the UV box plus 1 inch (about 13 inches total).

6. Cut the width of the cardboard insert so that two external tabs remain. This measurement will depend on the hypotenuse of the triangles cut from the UV box (see diagram at step 11).

7. Cut a rectangle out of the center of the insert.

Finished shape

8. Cut two 1-inch tabs at one end of the insert. Repeat steps 4–8 to make a second insert.

Slits are cut into the UV box at the corners of the triangular openings and at the back of the box so that the tabs of the inserts fit into the slits of the box.
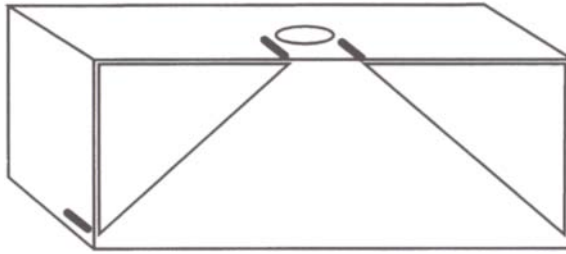
9. Cut four 1-inch slits into the UV box. Two of these slits are cut in the bottom of the box at the corners of the triangular openings, and one is cut at each end of the box. The width of the slit should accommodate the thickness of the cardboard insert.
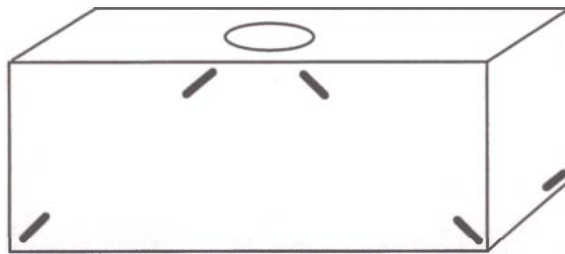
10. On each side of the back panel of the UV box, cut 1-inch slits that correspond to the corners of the triangles on the front panel of the box.

Place the inserts into the triangular openings of the UV box, as shown in the diagram.
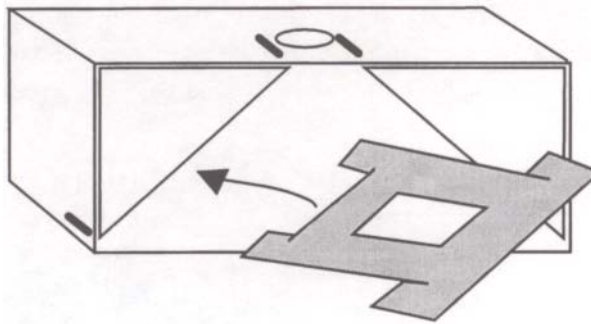
11. The inserts are placed inside the triangular openings of the UV box. The tabs of the inserts are pushed into the slits of the box.
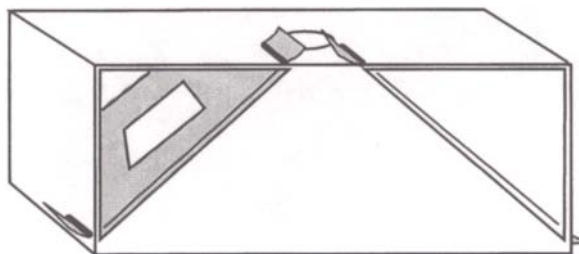
View from front
panel of the box

To eliminate light leaks, seal the box joins with electrical tape.



View from interior of the box
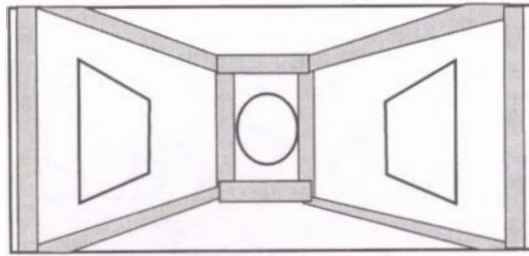
12. Using electrical or duct tape, seal all seams from the interior of the box.

Attachment B

**Photodocumentation Log Sheet**

Film roll no. _____

Name _____    Date _____

Subject _____

_____

_____

Lights _____    Correction filters _____

Camera _____    Lens _____

Meter _____    Polaroid _____

Diagram of equipment setup

Name _____

Film roll no. _____

| # | Sample | Camera distance | f-stop | Shutter | Filter | Description | Comments |
|---|---|---|---|---|---|---|---|
| 1 | | | | | | | |
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Protocol H

## Sample Application for Thin-Layer Chromatography

**Summary**

Application of the sample to the TLC plate using capillary micropipettes

Equipment:   Capillary micropipettes
             Pipette holder
             Prewashed, activated TLC plate, as specified for separation
             Spotting template or ruler
             Heat gun
Time:        Approximately 1 minute per sample; 10–20 minutes
             per TLC plate

**Scope**   The application of the sample to the TLC plate is an essential step in the separation of materials by means of thin-layer chromatography. In order to use the separation power of thin-layer chromatography, it is important for the sample to be accurately applied in a small compact spot. Samples can be applied manually using glass capillary micropipettes or a platinum-iridium needle. Samples can also be applied to a plate using specialized automated equipment. This protocol describes the use of disposable glass capillary micropipettes for the application of samples to the surface of the TLC plate.

## 1. Equipment and Supplies

The following equipment is needed for the application of a sample to a TLC plate using disposable glass capillary micropipettes:
1) Prewashed, activated TLC plate (as specified in each Protocol)
2) Capillary micropipettes (1.0 and 0.2 µl)
3) Micropipette holder
4) Spotting template or ruler
5) Heat gun

## 2. Samples

The general requirement is that the sample be dissolved in an appropriate carrier solvent. The sample solutions may be made from unknown, reference, or standard materials. The carrier solvent affects the spot size of the sample and the ease with which the sample can be applied. It is recommended that a relatively volatile solvent be used as the carrier solvent. Also, due to the solubility of various components within a sample, it is important that unknown and reference solutions be made with the same carrier solvent.

## 3. Sample Application Procedures

Before the sample solutions can be applied to a TLC plate, an appropriate plate must be selected, washed, activated, and marked. These procedures are described in detail in each material analysis protocol.

The application of a solution to a plate involves the filling of the micropipette with the solution and the transfer of the solution to the TLC plate. The spot diameter must be as small as possible since the separation zones tend to increase as the chromatogram develops. The surface of the stationary phase should not be pricked with the capillary micropipette; disturbances to the surface will affect the shape of the separation zones. *Gloves should be worn throughout the sample application procedure to avoid contamination of the sample or the TLC plate.* The samples are applied according to a scheme that alternates between reference and sample solutions to minimize systematic errors that might arise from the application of the solutions.
1) **Place the pipette into the holder**
   The micropipette holder is a glass tube with a dropper bulb attached to one end and a flexible fitting at the other. Check to see that the dropper bulb is attached near the end of the glass tube. Insert the micropipette into

the opening of the flexible fitting so that a small portion of the micropipette
(about 3 mm) can be seen inside the holder.
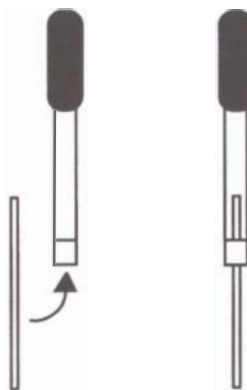


2) **Fill the pipette with the sample solution**
   Insert the free end of the micropipette into the sample solution. This is
   sometimes difficult, depending on the size of the vial in which the sample is
   stored. It can be facilitated by carefully tipping the vial so that the solu-
   tion flows along one wall of the vial. The micropipette should fill rapidly by
   capillary action. The meniscus of the solution can be seen traveling up
   the micropipette.
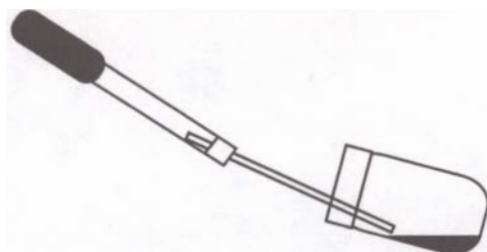


The vial can be tilted so that the
solution is more easily reached.                    Solution as it fills the pipette.
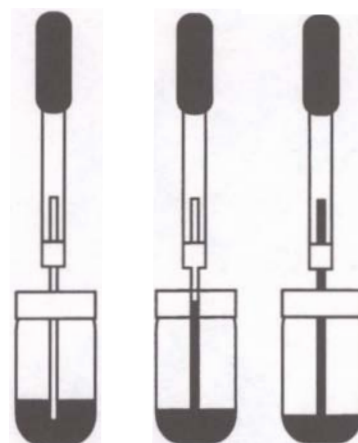
3) **Apply the sample solution to the TLC plate**
   The solution is applied to the surface in small increments (approximately
   0.5 µl in size). Transfer the sample solution to the TLC plate by placing the
   free end of the micropipette in contact with the surface of the TLC

plate at the tick mark of a lane. Hold the micropipette vertically and gently touch it to the surface of the TLC plate.
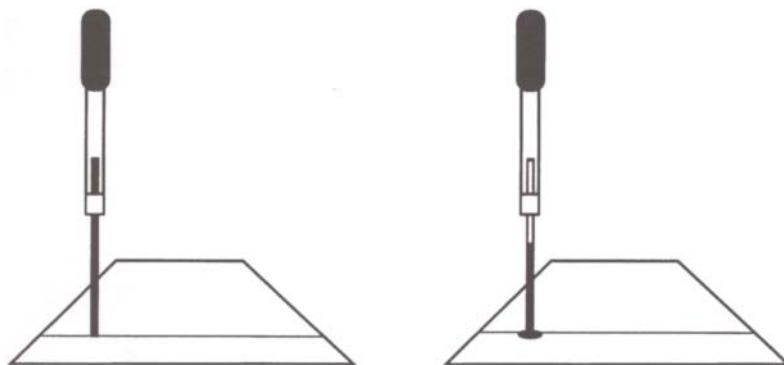


It is sometimes difficult to start the flow of the sample solution from the micropipette. If the flow does not start naturally, the solution can be gently forced from the micropipette. The dropper bulb contains a small hole. Cover the hole and very gradually apply pressure to the bulb. This should start the flow of the solution.

After a portion of the solution is applied, the spot is rapidly dried using a heat gun. The heat gun is set on a low, cool setting and is gently fanned back and forth across the spot. Care is taken to keep the micropipette away from the heat gun, as the sample solution could evaporate from the end of the micropipette. After the spot is dried, another portion is applied. This process continues until the micropipette is empty.
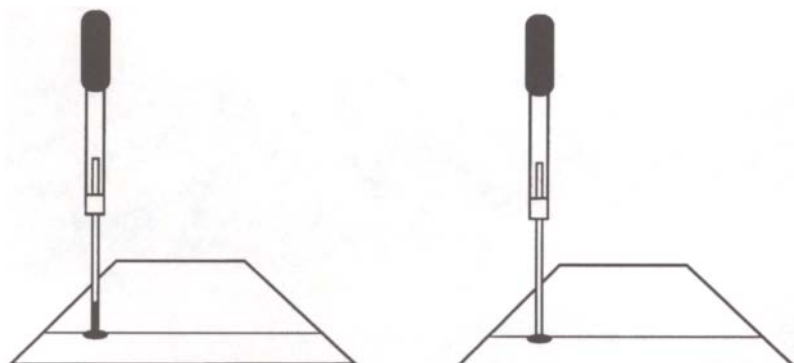
The solutions can be applied to the plate using the general application scheme below. Intersperse the unknown and reference solutions to minimize systematic error in the analysis.

## Scheme for Sample Application

R1, R2, etc. = reference solutions
U1, U2, etc. = sample solutions
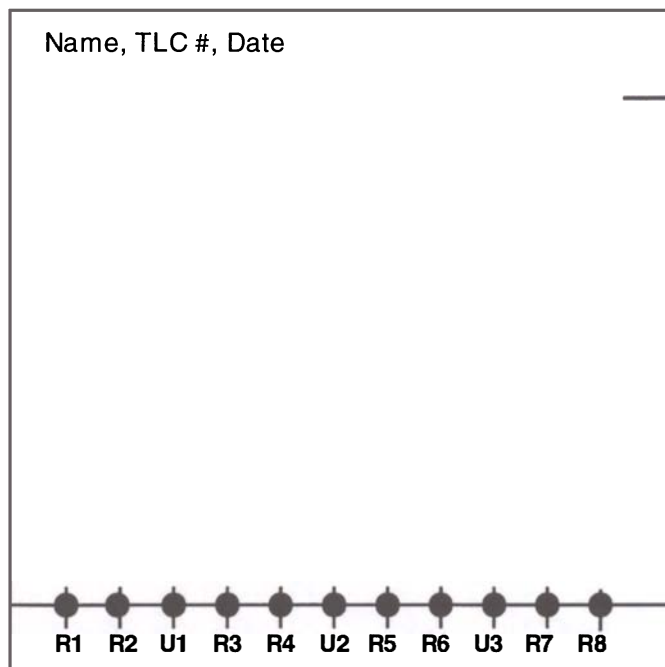
Protocol I

# Evaluation of a TLC Plate

**Summary**

Guidelines for the evaluation of a TLC plate

Supplies:    Lamp (tungsten or fluorescent bulb)

Ultraviolet lamp

Accurate ruler with metric (mm) markings

Calculator

**Scope**    A complete evaluation of a chromatogram aids in the documentation and interpretation of the analysis. The evaluation begins when the plate is removed from the developing chamber. The location and irregularities of the solvent front are marked. The plate is observed under visible light, and then under short-wave and long-wave ultraviolet light. After all visible and fluorescent areas are noted, the chromatographic plate is sprayed with a detection reagent for further visualization. Again, the plate is observed under visible and ultraviolet light. For each spot, the location (i.e., distance from the origin), color, and intensity are recorded. Next, $R_f$ values are calculated. This protocol describes the basic methodology for evaluating a chromatogram. For computer-aided evaluation, see **Protocol L**.

**Procedures**

1) **Mark the location and shape of the solvent front**
   Upon removal of the TLC plate from the development chamber, observe
   the location and shape of the solvent front of the plate. Using a pencil, lightly
   mark the front. Measure the distance between the origin and the solvent
   front ($d_s$) and record this value in the written documentation.

2) **Note any visible spots or areas under visible light**
   Visually examine the TLC plate, noting any colored spots or areas. If
   spots are seen, note the hue and value of the spot in the written documen-
   tation (see **Protocol E**). For instance, a spot may be yellow-orange (hue)
   and medium-dark (value).

3) **Note any fluorescent spots or areas under fluorescent light**
   Hold or prop a fluorescent lamp over the TLC plate about 2 inches from
   the surface. **Be sure to wear UV protective goggles during this examination
   to prevent possible eye damage**. Note any fluorescent spots.

4) **Spray and heat the TLC plate**
   Spray the TLC plate with the specified detection reagent and heat for the
   required length of time. Follow the instructions given in the individual
   protocols regarding the use of the detection reagent.

5) **Observe the TLC plate under visible light and note any changes**
   After detection with the appropriate reagent and heating, the plate is again
   studied under visible light. Faint discoloration or distinct spots may be
   seen on the plate. These are noted in the written documentation. They can
   be marked on the plate by circling with a pencil. If the plate is to be
   photographed, circling the spots is not recommended, as markings on
   the plate should be kept to a minimum.

6) **Observe the TLC plate under fluorescent light and mark the spots**
   The plate is again placed under an ultraviolet lamp and closely inspected. The
   number, color, and location of spots in each lane are noted. The spots can
   be marked if photographing of the plate is not planned. Otherwise, photog-
   raphy of the plate precedes any marking.

7) **Measure the distance from the center of each spot to the baseline
   of the TLC plate**
   Once the plate has been examined and photographed, the distance of each
   spot from the baseline is measured with an accurate metric ruler with
   millimeter markings. It is useful to circle the spots before measuring the
   location, particularly if they are fluorescent spots.

   There are two ways of determining the center of the spot. One way is to lay
   the ruler perpendicular to the baseline, along the lane, and estimate the
   center of the spot by eye. The second way is to measure the distance
   to the front edge of the spot ($d_1$) and the distance to the back edge of the
   spot ($d_2$). The center distance ($d_c$) is calculated from the average of the
   two distances:

   $$d_c = \frac{d_1 + d_2}{2}$$

This second method of determining the distance traveled by the spot may be more accurate, but it requires more time to process the information. Distances for each spot within a lane are tabulated in the written documentation.

8) **Calculate the $R_f$ value for each spot on the TLC plate**
Once the distances are tabulated, the distance to the solvent front is measured, $d_s$. The $R_f$ value is calculated as follows:

$$R_f = \frac{d_c}{d_s}$$

where $d_c$ is the distance that the spot traveled from the origin (see step 7 above), and $d_s$ is the distance traveled by the solvent front (see step 1 above).

9) **Compare the unknown sample to the reference materials that were chromatographed on the same TLC plate**
Examine the chromatographic patterns of each unknown and reference material. Compare the $R_f$ values of each material. The $R_f$ values should match within about 3% for a positive identification. Next, look at the color and intensity of each separation zone for both the unknown and the reference chromatograms. The final identification should take into consideration the $R_f$ value, color, and intensity of each separation zone. It is possible that the unknown sample may be a mixture of components. If this is the case, each separation zone must be carefully assigned to a component of the mixture.

10) **Document the TLC plate**
Following the visual examination of the TLC plate, written documentation is recorded following **Protocol E**. The plate is then photographed under normal or ultraviolet light (see **Protocols F** and **G**).

# Protocol J

## Acid Hydrolysis in a Pierce Vial Reaction Chamber

**Summary**

Acid hydrolysis of carbohydrate samples in preparation for TLC

Equipment:    Nitrogen gas

95 °C oven

Vacuum pump

Pierce glass vials (25 ml)

Miniert valves

Silicone rubber septa

22-gauge needle

Time:    30 minutes for preparation

5 hours for acid hydrolysis of carbohydrates

**Scope**    Some natural polymers (proteins, gums) need to be chemically broken down to individual components (amino acids, simple sugars) in preparation for thin-layer chromatography. For instance, proteins and carbohydrates require an acid hydrolysis step before the samples can be identified by means of TLC, as described in **Protocols A** and **B**. This protocol outlines an inexpensive alternative to the conventional acid hydrolysis that uses the relatively expensive Reacti-Therm equipment manufactured by Pierce.

The procedures described in this protocol can be used to prepare carbohydrate samples for TLC. **Protocol K** describes a vapor phase acid hydrolysis for the preparation of proteins for TLC.

The preparative steps for acid hydrolysis involve inspecting the Pierce vial reaction chamber and Miniert valve, checking the vacuum system, and adding acid to the sample vials. To provide a saturated acid atmosphere, a small volume of hydrochloric acid is also placed in the bottom of the vessel. After the sample vials are inserted into the Pierce vial, the Pierce vial is evacuated and flushed with nitrogen gas to remove the atmospheric oxygen that would oxidize the samples. The sealed and evacuated Pierce vial containing the binding media samples is then heated in a 90–95 °C oven until the acid hydrolysis is complete. At this time, the hydrolysates can be spotted on a TLC plate or refrigerated for later use.

**Scheme**

**Acid Hydrolysis in a Vacuum Desiccator**
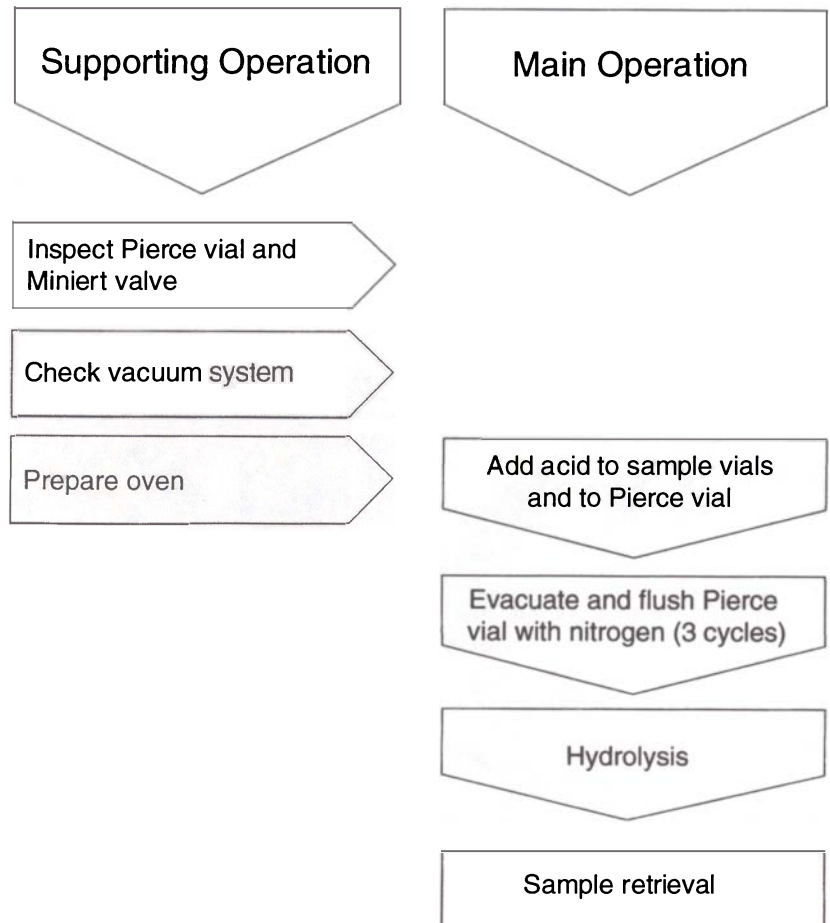
| Supporting Operation | Main Operation |
|---|---|

Inspect Pierce vial and Miniert valve

Check vacuum system

Prepare oven

Add acid to sample vials and to Pierce vial

Evacuate and flush Pierce vial with nitrogen (3 cycles)

Hydrolysis

Sample retrieval

## 1. Equipment and Supplies

The following equipment is needed for the preparation of acid hydrolysates of carbohydrate samples:
1) Pierce glass vials, 25 ml (no. 13074)
2) Pierce Miniert valves (no. 10130)
3) Pierce silicone rubber septa (no. 10153)
4) 1-ml sample vials
5) Vacuum pump
6) Nitrogen gas
7) Oven heated to 90–95 °C
8) Small glass beaker
9) 0.3$N$ HCl
10) Syringe (1 ml) and hypodermic needle
11) 22-gauge needle

## 2. Samples

The samples are weighed into 1-ml glass vials. Alternatively, taller vials with a smaller diameter (Pierce) can be used; this enables more samples to be packed into the reaction chamber. Although the vials should be capped after weighing to prevent sample loss or contamination, the caps must be removed once again for the acid hydrolysis procedure.

## 3. Preparation Procedures

Before the carbohydrate samples can undergo acid hydrolysis, the vacuum desiccator and oven must be prepared.
1) **Inspect the Pierce vials and the Miniert valves**
   The 25-ml glass vials from Pierce topped with the special Miniert valves are used as reaction chambers for the acid hydrolysis procedure and must be carefully inspected for flaws. The Teflon interior seal of the valve cap should be intact, the center bore should be cleared of any plastic bits left over from the machining process, and a new silicone rubber septum must be used for each hydrolysis. The threads of the glass vial should be smooth and have no cracks or losses. When the valve is in the "open" position (green tab is pressed in), a fine wire should be able to pass easily through the center bore.
2) **Check the vacuum**
   Connect the reaction chamber (Pierce vial with Miniert valve cap) to the vacuum line. Determine the optimal vacuum for the pumping system by noting the lowest vacuum that can be achieved when the Miniert valve is in the "closed" position (red tab is pressed in). Then open the valve to the "open" position and insert the 22-gauge needle into the bore of the valve. Adjust the tubing position or the position of the needle until the vacuum reaches the target value obtained for a closed system. This will be the desired vacuum and configuration when the reaction chamber is filled with the

samples. Leave the needle inserted into the bore of the Miniert valves, as the silicone rubber septum should optimally be pierced only once.

3) **Prepare the oven for hydrolysis**

The oven is stabilized at 90–95 °C for the acid hydrolysis procedure. To ensure even heating, the oven shelf is cleared of other materials. The reaction chamber will be positioned in the center of the oven.

### 4. Acid Hydrolysis Procedures

The acid hydrolysis of carbohydrate samples involves the addition of acid to the sample vials, the placement of a small volume of acid in the bottom of the Pierce vial reaction chamber, and three cycles of evacuating and flushing with nitrogen to remove residual oxygen. The evacuated reaction chamber is then heated in the oven for the number of hours appropriate for the samples (i.e., 5 hours for carbohydrates, **Protocol B**).

1) **Add acid to the sample vials**

Uncap the preweighed sample vials and carefully add 400 µl of 0.3$N$ HCl to each sample vial, using a syringe (1 ml) and needle.

2) **Place acid in the bottom of the Pierce vial**

Place 0.5 ml of 0.3$N$ HCl in the bottom of the Pierce vial. This solution will provide a saturated acid vapor environment during hydrolysis, and minimize the evaporation of acid from the sample vials.

3) **Place samples in the Pierce vial**

Carefully insert the sample vials into the Pierce vial reaction chamber with clean forceps. Cap the Pierce vial with the Miniert valve lid and tighten it firmly. (The needle should still be inserted in the center bore of the Miniert valve, from step 2 of the Preparation Procedures above.) For increased stability of the samples when the Pierce vial is handled or placed in the oven for hydrolysis, it is a good precaution to place the reaction chamber into a small beaker.

4) **Remove residual oxygen from the Pierce vial**

Slide the vacuum tubing onto the 22-gauge needle while the line is being flushed with nitrogen gas. Evacuate the Pierce vial until it reaches the target vacuum noted during the preparatory steps, and then flush the vial with nitrogen gas.

5) **Repeat evacuation and nitrogen flushing cycle**

To remove residual oxygen, repeat the evacuation and nitrogen flushing cycle a total of three times. After the final evacuation, close off the reaction chamber while the vacuum pump is still pumping on the system. Quickly and carefully withdraw the needle from the bore of the valve, and immediately close the Miniert valve. Turn off the pump and disconnect the vacuum tubing from the reaction chamber.

6) **Hydrolysis**

The evacuated Pierce vial reaction chamber can now be placed in the preheated oven for the duration of the hydrolysis (i.e., 5 hours for the acid hydrolysis of carbohydrates, **Protocol B**).

7) **Sample retrieval**

When the evacuated Pierce vial has been in the oven for the prescribed

length of time, it can be removed with oven mitts and set aside a few minutes to cool.

After the Miniert valve is carefully unscrewed and removed from the Pierce vial, the samples should be capped as soon as they are cool enough to handle.

Before analysis, evaporate the sample to dryness under a stream of nitrogen. The sample can be heated in a sand bath or a heating unit to 60 °C to facilitate the process. Reconstitute the sample with methanol (usually 400 μl). Store the samples in the refrigerator until used.

8) **Cleanup**

Dispose of the acid in the bottom of the Pierce vial by pipetting it into a large volume of water ($\geq$ 100 ml). Dispose of the dilute acid by pouring it down the sink under running water. Remove the silicone rubber septum from the Miniert valve and rinse the valve and vial in deionized water to remove traces of acid.

Protocol K

# Vapor Phase Acid Hydrolysis in a Pierce Vial Reaction Chamber

**Summary**

Vapor phase acid hydrolysis of protein samples in preparation for TLC

Equipment:   Nitrogen gas
             118–120 °C oven
             Vacuum pump
             Pierce glass vials (25 ml)
             Miniert valves
             Silicone rubber septa
             22-gauge needle

Time:        30 minutes for preparation
             24 hours for vapor phase acid hydrolysis of proteins

**Scope**  Some natural polymers (proteins, gums) need to be chemically broken down into individual components (amino acids, simple sugars) in preparation for thin-layer chromatography. For instance, proteins and carbohydrates require an acid hydrolysis step before the samples can be identified by means of TLC, as described in **Protocols A** and **B**. This protocol outlines an inexpensive alternative to the conventional vapor phase hydrolysis that uses the relatively expensive Reacti-Therm equipment manufactured by Pierce.

The procedures described in this protocol can be used to prepare protein samples for TLC. **Protocol J** describes an acid hydrolysis (liquid phase) for the preparation of carbohydrates for TLC.

The preparative steps for vapor phase acid hydrolysis involve inspecting the Pierce vial reaction chamber and Miniert valve, checking the vacuum system, and adding acid to the sample vials. After the sample vials are inserted into the Pierce vial, the Pierce vial is evacuated and flushed with nitrogen gas to remove the atmospheric oxygen that would oxidize the samples. The sealed and evacuated Pierce vial containing the binding media samples is then heated in a 90–95 °C oven until the vapor phase hydrolysis is complete. At this time, the hydrolysates can be spotted on a TLC plate or refrigerated for later use.

**Scheme**

**Vapor Phase Acid Hydrolysis in a Vacuum Desiccator**
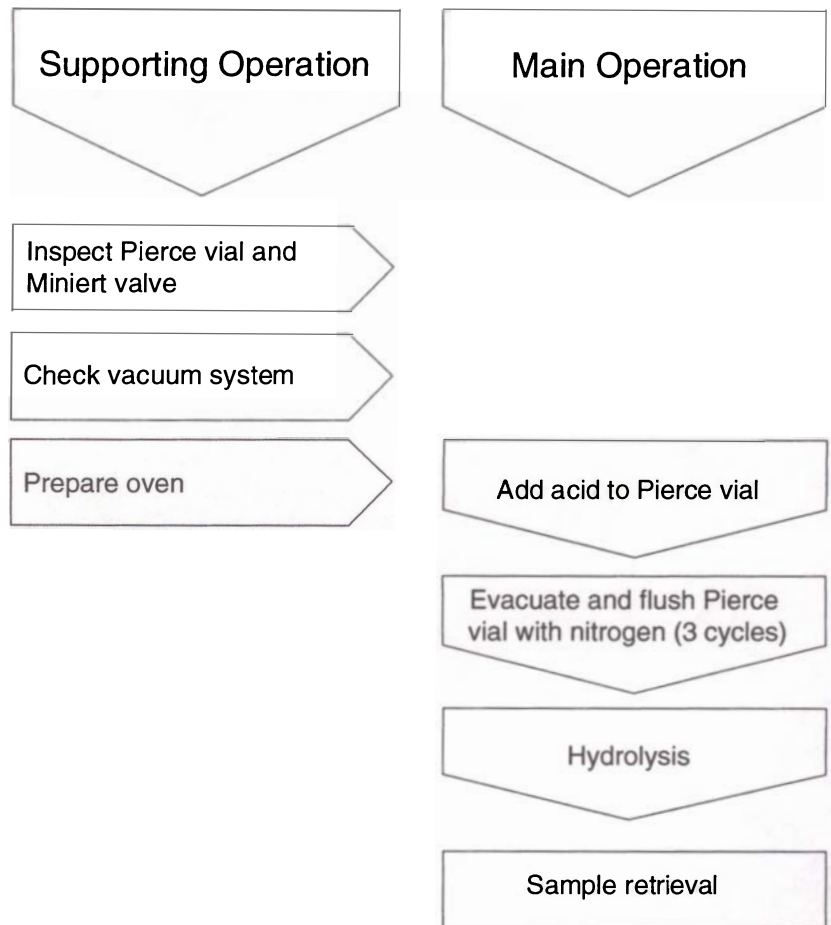
| Supporting Operation | Main Operation |
|---|---|

Inspect Pierce vial and Miniert valve

Check vacuum system

Prepare oven

Add acid to Pierce vial

Evacuate and flush Pierce vial with nitrogen (3 cycles)

Hydrolysis

Sample retrieval

1. **Equipment and Supplies**

The following equipment is needed for the preparation of acid hydrolysates of protein samples:
1) Pierce glass vials, 25 ml (no. 13074)
2) Pierce Miniert valves (no. 10130)
3) Pierce silicone rubber septa (no. 10153)
4) 1-ml glass vials
5) Vacuum pump
6) Nitrogen gas
7) Oven heated to 118–120 °C
8) Small glass beaker
9) 0.3$N$ HCl
10) Syringe (1 ml) and hypodermic needle
11) 22-gauge needle

2. **Samples**

The samples are weighed into 1-ml glass vials. Alternatively, taller vials with a smaller diameter (Pierce) can be used; this enables more samples to be packed into the reaction chamber. Although the vials should be capped after weighing to prevent sample loss or contamination, the caps must once again be removed for the vapor phase hydrolysis procedure.

3. **Preparation Procedures**

Before the protein samples can undergo vapor phase hydrolysis, the vacuum desiccator and oven must be prepared.
1) **Inspect the Pierce vials and the Miniert valves**
   The 25-ml glass vials from Pierce topped with the special Miniert valves are used as reaction chambers for the vapor phase hydrolysis procedure and must be carefully inspected for flaws. The Teflon interior seal of the valve cap should be intact, the center bore should be cleared of any plastic bits left over from the machining process, and a new silicone rubber septum must be used for each hydrolysis. The threads of the glass vial should be smooth and have no cracks or losses. When the valve is in the "open" position (green tab is pressed in), a fine wire should be able to pass easily through the center bore.
2) **Checking the vacuum**
   Connect the reaction chamber (Pierce vial with Miniert valve cap) to the vacuum line. Determine the optimal or target vacuum for the pumping system by noting the lowest vacuum that can be achieved when the Miniert valve is in the "closed" position (red tab is pressed in). Then open the valve to the "open" position and insert the 22-gauge needle into the bore of the valve. Adjust the tubing position or the position of the needle until the vacuum reaches the target value obtained for a closed system. This will be the desired vacuum and configuration when the reaction chamber is filled

with the samples. Leave the needle inserted into the center bore of the
Miniert valve, as the septum should optimally be pierced only once.

3) **Prepare the oven for hydrolysis**

The oven is stabilized at 118–120 °C for the vapor phase hydrolysis
procedure. To ensure even heating, the oven shelf is cleared of other mate-
rials. The reaction chamber will be positioned in the center of the oven.
*(Note: The oven should be kept at less than 122 °C because the stoppers on
the Miniert valves melt at higher temperatures.)*

## 4. Vapor Phase Hydrolysis Procedures

The vapor phase hydrolysis of protein samples involves the placement of a small
volume of acid in the bottom of the Pierce vial reaction chamber and three
cycles of evacuating and flushing with nitrogen to remove residual oxygen. *(Note:
Acid is not added to the sample vials.)* The evacuated reaction chamber is
then heated in the oven for the number of hours appropriate for the samples
(i.e., 24 hours for proteins, **Protocol A**).

1) **Place acid in the bottom of the Pierce vial**

Place 500 µl of *6N* HCl in the bottom of the Pierce vial. This solution will
provide a saturated acid vapor environment during hydrolysis, and minimize
the evaporation of acid from the sample vials.

2) **Place samples in the Pierce vial**

Uncap the preweighed sample vials and insert them carefully into the
Pierce vial reaction chamber with clean forceps. Cap the Pierce vial with the
Miniert valve lid, and tighten it firmly. (The needle should still be inserted into
the center bore of the valve, from step 2 of the Preparation Procedures
above.) For increased stability of the samples when the Pierce vial is handled
or placed in the oven for hydrolysis, it is a good precaution to place the
reaction chamber into a small beaker.

3) **Remove residual oxygen from the Pierce vial**

Slide the vacuum tubing onto the 22-gauge needle while the line is being
flushed with nitrogen gas. Evacuate the Pierce vial until it reaches the
target vacuum noted during the preparatory steps, and then flush the vial
with nitrogen gas.

4) **Repeat evacuation and nitrogen flushing cycle**

To remove residual oxygen, repeat the evacuation and nitrogen flushing cycle
a total of three times. After the final evacuation, close off the reaction
chamber while the vacuum pump is still pumping on the system. Quickly and
carefully withdraw the needle from the bore of the valve, and immediately
close the Miniert valve. Turn off the pump and disconnect the vacuum tubing
from the reaction chamber.

5) **Hydrolysis**

The evacuated Pierce vial reaction chamber can now be placed in the
preheated oven for the duration of the hydrolysis (i.e., 24 hours for the acid
hydrolysis of proteins, **Protocol A**).

6) **Sample retrieval**

When the evacuated Pierce vial has been in the oven for the prescribed length of time, it can be removed with oven mitts and set aside a few minutes to cool.

After the Miniert valve is carefully unscrewed and removed from the Pierce vial, add 400 µl of 0.1*N* HCl to each hydrolyzed sample. The samples should be capped as soon as they are cool enough to handle. Store the samples in the refrigerator until they can be analyzed.

7) **Cleanup**

Dispose of the acid in the bottom of the Pierce vial by pipetting it into a large volume of water (≥ 100 ml). Dispose of the dilute acid by pouring it down the sink under running water. Remove the silicone rubber septum from the Miniert valve and rinse the valve and the vial in deionized water to remove traces of acid.

Protocol L

## Semiquantitative Computer Analysis of a TLC Plate

**Summary**

Guidelines for the analysis of a TLC plate using Macintosh computer software

Equipment and supplies:   Macintosh Si computer with version 7.0 operating system or
greater (as much RAM as possible)
Handheld or flatbed scanner, or other means to digitize
the TLC plate
NIH Image v. 1.53 (public domain software)
Spreadsheet software (such as Excel)

**Scope**   Computer-aided evaluation of the TLC plate takes advantage of rapidly evolving
technology. First, an image of the TLC plate is digitized. Next, the image is
opened in NIH Image software, a public domain program. Each lane of the TLC
plate is scanned, and the pixel density of each lane is plotted with a gel plotting
macro that comes with the software. The distance from the origin and the
area of each peak is measured. The data are then assembled in a spreadsheet
program. Further research is in progress to investigate uses of statistical
software for pattern recognition and pattern matching of chromatograms. This
type of analysis is based on the Macintosh computer platform, and is intermediate
in cost between manual/graphical methods of evaluation (see **Protocol I**) and
conventional densitometry.

**Procedures**

1) **Digitize an image of the TLC plate**

   The image can be digitized in one of several ways. One way is to photo-
   graph the plate on slide or negative film, and have a photo CD made at the
   time of development of the film. Alternatively, if the chromatogram has
   resulted in colored spots, the plate can be scanned using a handheld
   scanner or a flatbed scanner. *If the image is scanned, it is recommended that
   it be scanned at 72 dpi. This creates a reasonably sized file that is easy to
   work with in the Image program.*

2) **Open the TLC plate image in NIH Image software**

   First, set the monitor to 256 colors option (go to the control panels under
   the Apple menu, open monitors, and select 256 colors). Next, find and open
   the NIH Image v. 1.53 software. Under the **File** menu, select the **Open**
   command and choose the image file to be analyzed. The image will appear
   in a new window on the screen.

3) **Rotate the TLC image**

   The image is rotated 90° (usually to the right) so that the origin of the
   plate is on the left side of the screen and the lanes are horizontal across the
   screen. Under the **Edit** menu choose **Rotate Right** command.

4) **Open the Gel Plotting Macro**

   A series of operations, called a macro, have been preprogrammed specif-
   ically for the analysis of the electrophoresis gels and can be used for
   the analysis of a TLC plate. The Gel Plotting Macro is located in a Macro
   folder inside the NIH Image folder. To use the macro, it must be
   opened. Under the **Special** menu, choose **Load Macro. . .** command. A
   window opens which shows the files that can be opened. Go to the Macros
   folder, and open the **Gel Plotting Macro** (the new one, not the old one).

5) **Mark the first lane**

   The mouse will turn to a *select* tool. Width and length of selected area are
   determined by marking the first lane. Draw a box from the origin to the
   solvent front that is the width of the first lane. To do this, place the select
   tool on the upper left corner of the first lane. Press down on the mouse
   button as you drag a rectangle over the lane. The width of the selected area
   can be adjusted only at the beginning of the analysis, so make sure that
   the width of this select box will be able to fit all of the lanes. Once you are
   satisfied with the selected area, go to the **Special** menu and select the
   **Mark First Lane [1]** command. A solid box will appear around the selected
   lane. A new window will appear containing the TLC image and the
   number 1 will appear next to the selected lane.

6) **Mark each of the next lanes**

   Using the mouse, move the selected area down over the next lane. To do
   this, place the mouse in the selected area (the cursor will change from a cross
   hair to an arrow) and hold down the button of the mouse as you drag it to
   the next lane. Once you have the selected area positioned over the next lane,
   go to the **Special** menu and select the **Mark Next Lane [2]** command.
   Again, a solid box will appear around the selected lane. Repeat this step
   until all of the lanes have been selected.

7) **Plot lanes**

Next, a TIFF file is generated that will contain a density plot for each selected lane. To generate this file, go to the **Special** menu and select the **Plot Lanes [3]** command. In a new window, a series of density plots will appear on a page. At this point, it is wise to save the plots. Under the **File** menu, select the **Save** command. Name the file and click on the **OK** button. To calculate the $R_f$ value, the distance traveled by the spot or spots and the distance traveled by the solvent front are needed. These values can be easily and quickly measured using NIH Image. Once the distances have been measured, the results can be exported to a spreadsheet for further analysis.

8) **Select the perimeter/length measurement function of the Image program**

Under the **Analyze** menu, select the **Options** submenu. Select the **Perimeter/length** measurement and deselect the **Area** measurement.

9) **Measure distance from origin to solvent front**

Choose the *line select* tool (which looks like a diagonal dotted line with a triangle underneath). Place the cross hair at the origin of the density plot, hold down the shift key, and drag it to the solvent front. Next, go under the **Analyze** menu and choose the **Measure** function. Note that the values window should show the count and the result of the measurement.

10) **Measure distance from origin to peak maximum for peaks in each lane**

Measure the distance traveled by the first peak by placing the cross hair on one end of the select line (the cross hair will turn into an arrow), hold down the shift key, and drag the select line down the page to the maximum point of the first peak. Go to the **Analyze** menu and choose the **Measure** function. The count should change in the values window. Continue to take measurements for all peaks. Care should be taken to note the order with which the peaks are measured.

Next, go to the **Analyze** menu and choose the **Show Results** command. A new window will appear that indicates the measurement count in the first column and the length in the second column. Print results by selecting **Print Measurements** command under the **File** menu. These results can be exported to a spreadsheet or saved from NIH Image.

11) **Calculate the $R_f$ value for each spot on the TLC plate**

Once the distances are tabulated, the $R_f$ value is calculated as follows:where $d_c$ is the distance that the spot traveled from the origin (see step 10 above) and $d_s$ is the distance traveled by the solvent front (see step 9 above). The $R_f$ value can be easily calculated in the spreadsheet program Excel.

12) **Compare the unknown sample to the reference materials that were chromatographed on the same TLC plate**

Examine the chromatographic patterns of each unknown and reference material. Compare the $R_f$ values of each material. The $R_f$ values should match within about 3% for a positive identification. Next, look at the color and intensity of each separation zone for both the unknown and the reference chromatograms. The final identification should take into consideration the $R_f$ value, color, and intensity of each separation zone. It is possible that the unknown sample may be a mixture of components. If this is the case, each separation zone must be carefully assigned to a component

of the mixture. NIH Image can also be used to calculate the peak area for each peak in the chromatogram. A baseline is drawn by the operator for each peak. The baseline for unresolved peaks must be estimated. The w*and* tool is then used to select the peak. This method is still in developmental stages. The following steps may be improved in the future.

13) **Select the Perimeter/length measurement function of the Image program**
Under the **Analyze** menu, select the **Options** submenu. Select the **Perimeter/length** measurement and deselect the **Area** measurement.

14) **Draw a baseline for each peak**
Select the *line* tool. Draw a baseline for any peak that does not touch the existing baseline. To do so, place the cross hair on the left side of the peak and hold down the mouse button while drawing the line to the right side of the peak. Repeat this step until all peaks have a baseline.

15) **Measure area for each peak**
Select the *magic w*and* tool from the tools window (it looks like a wand and is the ninth tool in the first column). Place the wand end of the tool in the center of the peak and click once on the mouse. A running stripe will surround the selected area, and the measured area will appear in the values window.

In addition to tabulating the areas, the value of the area can be placed on the plot by selecting the *type* tool (a big A) and placing it in the image. Choose where you want the value on the plot, then hold the option key and click once on the mouse button. The last measured value will automatically be placed on the plot.

Again, care should be taken to keep track of the order in which the peaks are measured. The measurements are viewed by selecting **Show Results** from the **Analyze** window. Print results by selecting **Print Measurements** command under the **File** menu. These results can be exported to a spreadsheet or saved in NIH Image.

# Glossary

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| **accuracy** | agreement between an experimental result (a single measurement or the mean of several replicate measurements) and the true or theoretical value |
| **activation** | process of heating an adsorbent layer to drive off moisture; the sorbent is converted to its most retentive and receptive state |
| **activity grades** | standard grading system (Brockmann activity grades) for the activity (adsorptivity) of alumina base upon deactivation with water; Grade I is anhydrous alumina and has the highest activity; Grades II, III, IV, and V contain 3%, 6%, 10%, and 15% (by weight) water, respectively |
| **adsorbate** | an adsorbed substance |
| **adsorbent** | a substance (usually solid) that adsorbs another substance |
| **adsorption** | phenomenon of surface adhesion (as opposed to absorption); adhesion in an extremely thin layer of molecules to the surfaces of solid bodies or liquids with which they are in contact |
| **adsorption chromatography** | process whereby the components of a sample are separated by interaction between adsorptive forces of a medium (stationary phase) and a solvent (mobile phase) |
| **alumina** | common adsorbent ($Al_2O_3$) |
| **analyte** | solute that is to be identified or, more often, quantitatively determined by TLC or other method |
| **argentation TLC** | TLC employing silver nitrate impregnated in the layer material, usually silica gel; this impregnation changes the separation characteristics of the silica gel |
| **ascending chromatography** | chromatography in which the mobile phase moves upward in the medium |
| **band** | chromatographic zone; region where the separated substance is concentrated |
| **bed** | column or layer of porous material of the stationary phase, the interstices being filled with the mobile phase |
| **binder** | any chemical added to a sorbent to improve the stability or hardness of the layer |
| **binding medium** | natural or synthetic material used in paints to hold pigment particles together and adhere the pigmented layer to the painting substrate |
| **bonded phase** | stationary phase chemically bonded to (as opposed to mechanically deposited on) a support material |
| **capacity factor ($k$)** | a measure of sample retention by a layer: $$k = \frac{\text{mass of solute/unit of stationary phase}}{\text{mass of solute/unit of mobile phase}}$$ |
| **cellulose** | common medium for separation on a TLC plate |

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| **chamber** | tank, jar, or vessel in which chromatographic separation takes place |
| **chromatogram** | a series of separated bands of zones in the stationary phase; the end product of the chromatography process |
| **chromatographic solvent** | solvent or mixture of solvents used as the mobile phase |
| **chromatographic system** | combination of the solvent, the sorbent, and components of the sample mixture; the interactions between the components of the system determine the selectivity of the separation |
| **chromatography** | a method of analysis in which the flow of a mobile phase (gas or liquid) promotes the separation of substances by differential migration from a narrow initial zone, in a sorptive medium |
| **chromatoplate** | a thin-layer plate; a layer of sorbent coated on a solid support such as glass, aluminum, or plastic |
| **continuous development** | development occurring over a distance that is usually greater than one plate length; development is expressed as a function of time rather than distance |
| **deactivation** | process of making the chromatographic layer less active to decrease its separation capabilities; occurs in the presence of water (see activation) |
| **demixing** | process where a mixed solvent system separates into phases, such as water separating from acetonitrile in an acetonitrile : water (80:20) solution; will result in secondary fronts on the TLC plate |
| **densitometry** | measurement of a zone on a layer with an instrument that determines the optical density of the zone; this enables the determination of the quantity of a spot |
| **derivatization** | reaction of solutes before chromatography (or directly on the layer) for the purpose of facilitating separation or detection |
| **descending chromatography** | chromatography in which the mobile phase moves downward on the plate |
| **destructive detection** | detection process that changes the chemical nature of the substance being detected in an irreversible manner (i.e., sulfuric acid charring) |
| **detection** | a way of seeing and quantifying zones; process of locating a separated substance on a chromatogram, whether by physical methods, chemical methods, or biological methods |
| **developing solvent** | mobile phase |
| **development** | flow of mobile phase through the chromatogram to cause separation of the components of the sample |
| **efficiency** | quality of the separation; an efficient layer produces compact zones; more specifically, the efficiency of a separation is indicated by the narrowness of a zone (or a TLC spot) compared to its distance of migration |
| **eluent** | solvent that removes adsorbed material from an adsorbent |
| **eluotropic series** | series of solvents or solvent mixtures arranged in order of eluting power |
| **eluting power** | measure of the ability of the solvent to transport a solute through a chromatographic system |
| **elution** | removal of a solute from a sorbent by washing with a suitable solvent |
| **equieluotropic** | solvents with equal eluting power |
| **flatbed chromatography** | planar chromatography; thin-layer chromatography is a form of planar chromatography |
| **fluorogenic** | reagent or reaction causing a solute to become fluorescent |

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| **front** | visible boundary at the junction of the mobile-phase wetted layer and the "dry" layer |
| **gradient elution** | development using a solvent system that is changed in a continuous or stepwise mode to effect separation; normally done to increase the strength of the eluent |
| **hard layer** | abrasion-resistant sorbent layer bound to the backing by an organic polymer |
| **homologue** | member of a homologous series (i.e., a series in which each successive member contains an additional -$CH_2$- group); methanol, ethanol, and propanol are homologous alcohols |
| **HPLC** | high-performance liquid chromatography |
| **$hR_f$** | 100 x $R_f$ |
| **hydrophilic** | substances that are soluble in water or other polar solutions |
| **hydrophobic** | substances that are soluble in nonpolar solvents and insoluble in water |
| **impregnation** | loading of the sorbent with a liquid or a solid to change the chromatographic behavior of the layer; an example is $NaNO_3$ impregnated silica gel |
| **in situ** | occurring in place (e.g., on the thin layer) |
| **ion exchange** | a competitive process whereby ions of the same charge replace each other in a given phase |
| **isocratic** | a nongradient chromatographic system; no change in solvent strength |
| **ligand** | a group, ion, or molecule coordinated to a central atom in a complex |
| **lipophilic** | having an affinity for lipids; hydrophobic (nonpolar) |
| **mass transfer** | movement of a solute between the stationary and mobile phases |
| **microgram (μg)** | $1 \times 10^{-6}$ g or 1000 ng |
| **migration** | travel of sample in the medium in the direction of the mobile-phase flow |
| **mobile phase** | moving phase (liquid or gas) of a chromatographic system |
| **multiple-development chromatography** | chromatography repeated a number of times using the same or different mobile phases |
| **nanogram (ng)** | $1 \times 10^{-9}$ g or 0.001 μg |
| **nondestructive detection** | detection of a substance on a chromatogram by a process that will not permanently change the chemical nature of the substance being detected; visualization with UV light or with iodine vapor are examples of nondestructive methods |
| **normal phase TLC** | adsorption or partition TLC in which the stationary phase is more polar than the mobile phase |
| **origin** | initial position of the applied sample on the chromatographic plate |
| **partition chromatography** | process in which sample is partitioned between two immiscible liquid phases (as in TLC), or between a gas and a liquid (as in gas chromatography); separation occurs because one phase is stationary while the other is mobile |
| **partition coefficient or ratio ($K_d$)** | ratio of concentration of solute after partition between two immiscible phases: $$K_d = C_s/C_m$$ where $C_s$ and $C_m$ are the concentrations in the stationary and mobile phases, respectively |
| **planar chromatography** | common term for thin-layer or paper chromatography; also known as flatbed chromatography |
| **polar** | polarity indicates partial positive and partial negative charges on different parts of the molecule |

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| **polar mobile phase** | mobile phase consisting of polar molecules (e.g., water or ethyl acetate) |
| **polar stationary phase** | stationary phase consisting of polar molecules (i.e., silica gel) |
| **precision** | measure of the agreement of replicate analyses; not a measure of accuracy |
| **preparative layer chromatography (PLC)** | used for the separation of larger amounts of substance than are normally separated with analytical TLC; normally a thicker layer (500–2000 μ) or sorbent is employed than in TLC |
| **radial (circular) development** | development of a layer in such a manner as to form circular or arc-shaped solute zones |
| **resolution** | measure of the quality of separation between two substances |
| **reversed phase** | chromatography with a stationary phase that is less polar than the mobile phase; usually applies to TLC with an aqueous mobile phase and a bonded nonpolar stationary phase |
| **$R_f$ value** | the distance from the origin to the center of the separated zone divided by the distance from the origin to the solvent front: |

$$R_f = \frac{\text{distance traveled by solute}}{\text{distance traveled by solvent front}}$$

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| **sandwich chamber** | developing chamber formed from the plate itself, a spacer, and another uncoated cover plate that stands in a trough containing the mobile phase |
| **saturation** | condition of a chamber that is lined with paper and equilibrated with mobile-phase vapors before beginning chromatographic development |
| **secondary front** | an additional mobile-phase front that occurs behind the primary solvent front due to the phenomenon of demixing |
| **selectivity** | ability of a chromatographic system to resolve the components of a mixture |
| **sensitivity** | ability to detect or measure a small mass of analyte |
| **separation zone** | *see zone* |
| **silica gel** | silicic acid; the most widely used sorbent for TLC |
| **soft layer** | sorbent layer prepared without binder or with gypsum binder (*see hard layer for comparison*) |
| **solvent** | liquid(s) used for mobile phase; not identified a priori with mobile phase |
| **solvent front** | *see front* |
| **sorbent** | a generalized term for the chromatographic stationary phase; a general term for the sorbent layer on a TLC plate |
| **sorption** | general term for the attraction between a sorbent layer on a TLC plate and a solute, without specification of the type of physical mechanism involved (i.e., adsorption, partition, ion exchange, or a combination of these) |
| **spot** | used synonymously with zone, but usually meant to indicate a round or elliptical shape |
| **stationary phase** | phase of the chromatographic system that remains stationary (i.e., the silica gel of the silica gel plate or a bonded phase, such as C18) |
| **stepwise elution** | gradient elution in a stepwise mode |
| **streak** | *see tailing* |
| **support** | sheet of glass, plastic, or aluminum coated with the TLC sorbent; gives physical strength to the layer |

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| **tailing** | comet-shaped spots; elongated spots that indicate incomplete separation; this situation should be avoided |
| **theoretical plate number ($N$)** | measure of the efficiency of a chromatographic system |
| **two-dimensional development** | successive development of a chromatogram in directions orthogonal to each other, with a different mobile phase used for each of the two developments; a plate is first developed in one mobile phase, then dried, turned 90°, and developed in a second mobile phase |
| **unsaturated** | conditions under which a plate is run in a chamber without presaturation; usually yields different results than with a presaturated chamber |
| **visualization** | detection of the zones on a chromatogram |
| **zone** | area of distribution on the layer containing the individual solutes or mixture before, during, or after chromatography; the initial zone is the applied sample prior to development; *band*, *zone*, and *spot* are used more or less interchangeably, but *spot* usually denotes a round zone and *band* a flat, horizontally elongated zone |

## About the Authors

Mary F. Striegel graduated from the University of Louisville in 1981 with a B.A. in fine arts and chemistry. She received her M.S. in analytical chemistry from Indiana University–Purdue University in Indianapolis and completed her Ph.D. in inorganic chemistry in 1988. She was an assistant scientist at the Getty Conservation Institute for six years before joining the staff of the National Center for Preservation Technology and Training as a materials scientist in 1995. Her research includes the application of new analytical methods to the examination of artist materials. Her interests include technical photography and acoustic microscopy.

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