DENSITOMETRY IN THIN LAYER CHROMATOGRAPHY

Practice & Applications

Densitometry in Thin Layer Chromatography

PRACTICE AND APPLICATIONS

Edited by

JOSEPH C. TOUCHSTONE
University of Pennsylvania

JOSEPH SHERMA
Lafayette College

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Contributors

M. Amin
Schering Corporation
Berlin, Germany

T. Aratani
Hiroshima University
Hiroshima City, Japan

Y. Asakawa
Hiroshima University
Hiroshima City, Japan

P. Bauman
University of Lausanne
Prilly-Lausanne, Switzerland

K. Brain
The Welsh School of Pharmacy
Cardiff, Wales

G. Chen
Hoffman-LaRoche Inc.
Nutley, New Jersey

D. A. Cook
Mead Johnson Research Center
Evansville, Indiana

R. Cox
Burma-Castrol Company
Berkshire, England

C. Crowley
Hoffman-LaRoche Inc.
Nutley, New Jersey

R. L. DeAngelis
Burroughs-Wellcome Research
Laboratories
Research Triangle Park
North Carolina

A. Der Marderosian
Philadelphia College of
Pharmacy and Science
Philadelphia, Pennsylvania

M. F. Dobbins
University of Pennsylvania
Philadelphia, Pennsylvania

D. T. Downing
Boston University
Boston, Massachusetts

P. Duke
Hoffman-LaRoche Inc.
Nutley, New Jersey

P. H. Fishman
National Institutes of Health
Bethesda, Maryland

J. H. Fleischer
University of Arizona
Tucson, Arizona

J. G. Heathcote
University of Salford
Salford, England

J. P. Heotis
Norwich Pharmacal Company
Norwich, New York

H. K. L. Hunt
University of the Orange
Free State
Bloemfontein, South Africa

H. Hutchinson
Hoffman-LaRoche Inc.
Nutley, New Jersey

M. Kaykaty
Hoffman-LaRoche Inc.
Nutley, New Jersey

A. MacDonald
Hoffman-LaRoche Inc.
Nutley, New Jersey

J. D. Manes
Mead Johnson Research Center
Evansville, Indiana

M. J. Mantel
Municipal Hospital
Rotterdam, The Netherlands

J. F. Martin
University of Salamanca
Salamanca, Spain

S. R. Max
University of Maryland
Baltimore, Maryland

L. E. McDaniel
Rutgers University
New Brunswick, New Jersey

J. L. Mertz
Norwich Pharmacal Company
Norwich, New York

S. A. Narang
National Research Council
of Canada
Ottawa, Canada

Z. J. Petryka
Northwestern Hospital
Minneapolis, Minnesota

V. Pollak
University of Saskatoon
Saskatoon, Canada

A. Popick
Hoffman-LaRoche Inc.
Nutley, New Jersey

R. H. Quarles
National Institutes of Health
Bethesda, Maryland

R. Saperstein
Hoffman-LaRoche Inc.
Nutley, New Jersey

D. L. Schneider
Mead Johnson Research Center
Evansville, Indiana

H. Seiler
University of Basel
Basel, Switzerland

J. Sherma
Lafayette College
Easton, Pennsylvania

C. W. Sigel
Burroughs-Wellcome Research
Laboratories
Research Triangle Park
North Carolina

L. L. Smith
University of Texas
Galveston, Texas

E. Songu
University of Pennsylvania
Philadelphia, Pennsylvania

R. D. Stubblefield
United States Department
of Agriculture
Peoria, Illinois

J. M. Steyn
University of Orange Free
State
Bloemfontein, South Africa

J. I. Teng
University of Texas
Galveston, Texas

J. C. Touchstone
University of Pennsylvania
Philadelphia, Pennsylvania

L. R. Treiber
Case-Western Reserve University
Cleveland, Ohio

J. Westheimer
Hoffman-LaRoche Inc.
Nutley, New Jersey

J. L. Woolley, Jr.
Burroughs-Wellcome Research
Laboratories
Research Triangle Park
North Carolina

B. Wortmann
W. Wortmann
Universitäts Frauenklinik
Mainz, Germany

R. Wu
National Research Council
of Canada
Ottawa, Canada

Preface

Thin layer chromatography (TLC) has shown a phenomenal growth in recent years. Particularly instrumental in this growth has been the ready availability of uniform layers with which reproducible results can be obtained. With improved understanding of the theories of chromatography and the factors that affect the results, it has become increasingly used in analytical chemistry. Very prominent in this growth is the advent of densitometers that have been designed for the scanning of thin layer chromatograms for quantitative purposes.

The utility and success of densitometry (either absorbance or fluorescence) of thin layer chromatograms as a quantitative method is due to both the simplicity and rapidity as well as the sensitivity of the methodology. When one considers that as many as 15 samples can be analyzed on a single 20×20 cm thin layer chromatogram that can be scanned by densitometry in as little as 30 minutes, one can easily realize the practical applications of this method, which can be applicable in many instances when fluorescent compounds are analyzed at the parts per billion (ppb) level. This technique is also rapid in that it is easy to change conditions of the chromatography, in either the activity or the nature of the layer as well as the nature or strength of the mobile phase.

The organization of this text is based on the use of densitometry as a means to assess quantities of separated materials in scanning thin layer chromatograms. It is structured for those who have some understanding of or have done, TLC. Our aim was to keep theory at a minimum and to stress applications. Consequently, the beginning of the book is limited to presenting factors that contribute to the success of the separations, which in turn affect the success of the scanning by densitometry. As is true of all analytical instruments, if one has a poor chromatogram, the densitometer will indicate just that. Too many workers are prone to blame the instrument without realizing that the fault lies in poor chromatographic technique. However, knowledge of the instrument parameters also contributes to the success of the experiment.

With a background and knowledge of the proper characteristics of the chromatograms and of instrumentation available, one can

develop sensitive and rapid analytical methodology. The instrumental characteristics and general guidelines for densitometry are covered in the beginning chapters, and the bulk of the text then covers applications for various types of compounds. No attempt has been made to wean the authors from their various methodologies in the use of the densitometer. Rather, each author has been allowed to present his method as it applied to his own particular problem. Also, within limits, some latitude in style has been permitted, but generally the same outline has been followed. Consequently, there are a number of methods that perhaps would only succeed with the compounds in question. By the same token, anyone reading this volume will probably find pearls of technique that perhaps will help him solve his own particular problem without having any bearing on the compound class. Specialized areas, in which the literature is very scattered and data collection and evaluation are important, are covered. Thus the book can serve as a reference as well as an experimental guide.

One chapter departs from the general purpose of the book, in that it does not deal with densitometry. The chapter on planimetry of bioautograms (Chapter 9) is included because no available method involving scanning appears to be available and because the detection procedure is considered too important to ignore completely.

No book can be compiled without the cooperation of a wide variety of disciplines and the many people involved. This represents truly an international volume and attests to the widespread popularity and utility of the technique of densitometry of thin layers as an analytical tool. We appreciate the patience and cooperation of all involved. We also fully appreciate the support of our home departments, which enabled us to succeed in completion of this undertaking.

Joseph C. Touchstone
Joseph Sherma

Philadelphia, Pennsylvania
Easton, Pennsylvania
July 1978

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PRACTICE

CHAPTER 1

History of In Situ Scanning

J. C. Touchstone

1.1 INTRODUCTION

Scanning of "thin layers" or *in situ* densitometry as discussed in this book involves direct measurement of absorptive properties of a substance separated on a "thin film." Here we are primarily involved in scanning of thin layer chromatograms. This includes the quantitation of fluorescent compounds as they fall in the class mentioned in the first sentence. Fluorescence must be preceded by absorption. Early methods include that of Block (1) and Bull et al. (2) who used scanning *in situ* of paper chromatograms for quantitation of amino acids. Much of this work was described in the book *Paper Chromatography and Paper Electrophoresis* by Block et al. (3).

1.2 EARLY HISTORY

Quantitation *in situ* of "thin film" chromatograms predates the beginning of modern thin layer chromatography. Other attempts to quantitate materials separated on chromatograms, without removal from the layer, appear also to be in the direct scanning of paper chromatograms of amino acids. Patton and Chism (4) used a Welch "Densichron" for scanning of paper chromatograms of amino acids. The strip was manually drawn through a specially made paper guide. They stressed the importance of the standard curve for use in quantitative assessment of the separated amino acids. McFarren et al. (5) reported in 1951 the use of a Photovolt "electron emission densitometer" for quantitative assessment of carbohydrates separated on paper chromatograms. They used only the maximum density of the spot and claimed an error of no greater than 5%.

In 1951 Tennant et al. (6) described a paper strip guide which was designed to be used in the cell compartment of the standard Beckman DU spectrophotometers. Strips were cut from the chromatograms, which were then wound on a spindle to be fed across an aperture facing the light source. The strip was guided manually, after balancing the instrument on a blank area, and the optical density plotted against distance on the chromatogram. This gave a pattern of peaks of separated zones on the chromatogram. This technique was used extensively by Touchstone et al. (7, 8) in their work on the quantitation of urinary corticosteroids separated by paper chromatography. These examples, perhaps, are the forerunners of *in situ* densitometry of thin layer chromatograms. There are many others, but paper chromatography is not in universal use now as it was at that time.

The instruments used in the early attempts to scan for quantitation *in situ* on paper and thin layer chromatograms were designed for the scanning of gels. Modifications were added in order to adapt them for scanning thin layer chromatograms. *In situ* scanning by densitometry can be a quick and reliable method for assessing the amount of a substance directly on the chromatogram. However, it is necessary for the operator to take the effort to reproducibly prepare the chromatograms, since the precision of the results depends more on this than on the operation of the instrument itself.

Densitometry *in situ* on chromatograms represents direct determination of the concentration by a form of absorptiometry. The chromatogram is scanned by a beam of light of predetermined wavelength, and the proportion of this either reflected or transmitted by the layer is measured photoelectrically. In this way this is similar to conventional absorptiometry, and it might be considered that a relationship similar to Beer's law might apply.

The majority of the early commercially available instruments (1950-1960) gave regression lines obeying Beer's law. This follows when the medium is transparent. Paper and thin layer chromatograms are, at best, truly translucent. Kubelka and Munk (9, 10) investigated the relationship between optical density and absorptivity on solid media. Two expressions were derived, one for reflectance and the other for transmittance. Reflectance could be related to the coefficient of absorption and coefficient of scatter.

In transmission, the thickness of the layer must be considered. Thus, a more complicated expression results. Present theoretical considerations in densitometry of thin layer chromatograms are based on expressions of the Kubelka-Munk theories. Franglen (11) recommended oiling of paper chromatograms prior to densitometry in order to cut down scatter or to render the chromatograms more transparent for transmittance evaluation. About this time, the use of photographs of thin layer chromatograms for absorptive scanning was developed. However, this method was indirect and time consuming.

In 1960, Hefendehl (12) described a method to make thin layer chromatograms transparent by spraying with paraffin-ether (1:1 v/v). After photoprinting, quantitation was accomplished by absorptimetric means using the photograph. Suitable densitometers were not then available, so the photograph was cut into strips. The method of Squibb (13), described in the next paragraph, was another attempt to use the available densitometers.

Silica gel was spread on the matted side of clear plastic sheets. After spotting and separation of amino acids, the chromatogram was sprayed, then Tuffilm spray was applied. After drying, the chromatogram had the appearance of an acetate film which could be cut into strips and scanned in standard spectrophotometers. After separation of amino acids and spraying with ninhydrin, the plates were scanned in a Photovolt densitometer. The method was used to quantitate free amino acids in avian liver (14).

These probably were attempts to satisfy some of the requirements set forth in the Kubelka-Munk equation in regard to scatter. There have been a number of other papers describing the use of scanning of photographs for quantitation of components separated on thin layer chromatograms. Even as late as 1970, workers were using the photographic method. Kelleher (15) described a method involving Polaroid Type 46-L projection film, a Polaroid Land camera, and a conventional thin membrane densitometer (Analytical Recording Densitometer-Integrator of Beckman Instruments). Peak areas from the scans were measured by planimetry.

Early attempts to apply *in situ* densitometry to thin layer chromatograms appear in the report of Blank et al. (16). These

investigators extensively studied the analysis of glycerides by thin layer chromatography followed by charring with sulfuric acid. The chromatograms were then scanned directly with a Photovolt densitometer. They showed that the area of the peaks in the scanning records was proportional to the amount of material in the spot. The peak areas were specific for each compound. Consequently, a calibration curve was required for each compound. The technique was used for determination of saturated and unsaturated fatty acids in glycerides (17). These authors have done considerable work on the *in situ* densitometry of chromatograms after charring. As early as 1963, Photovolt provided its model 52C with an accessory to handle 20×20 cm plates.

About the same time, Joyce-Loebl had available an accessory for evaluation of thin layers using its Chromoscan densitometer. Barrett et al. (18) used this instrument in their early work on determination of lipids after charring with sulfuric acid. These determinations were done in the reflectance mode in contrast to those wherein transmittance was evaluated with the Photovolt instrument. Jork (19) also used the Joyce-Loebl and the accessory in his early work with quantitative thin layer chromatography (TLC).

The methods so far mentioned involved densitometry of colored zones either inherent in the compound or induced by charring or derivative formation. Mention must be made of the use of fluorodensitometry for *in situ* assay of fluorescent compounds separated on thin layer chromatograms, since some of the early work in TLC involved this type of quantitation.

Fluorescence of decomposition products of carbohydrates was evaluated by a Turner fluorometer by Connors and Boak (20). Using a Photovolt densitometer with the fluorescence accessories, Fischer and Rigelman (21) were able to quantitate griseofulvine by assessment of fluorescence, using the thin layer scanning attachment of Camag for the Turner fluorometer. (For more detailed description of this and other instruments mentioned in this chapter, see Chapter 5 on instrumentation.) Sawicki and co-workers (22-24) pioneered the use of direct fluorescence determination for quantitation of polycyclic hydrocarbons from air after separation on thin layer chromatograms. They used the Aminco-Bowman spectrofluorometer with the accessory TLC plate scanner.

Frei and co-workers (25) had investigated reflectance spectra of dyes separated on alumina. Seiler and co-workers (26) have done extensive work on the determination of tryptamine and beta-indoyl acetic acid using fluorescence measurements carried out with the Zeiss attachment. Blunden and co-workers (27) used a "Vitatron" densitometer to estimate diosgenin in Dioscorea.

1.3 ADVENT OF DENSITOMETERS

Jork, in 1966, described the makeup of the accessory eventually to be made commercially by the Zeiss organization (28). The method was based on measurements of directional reflectance and made it possible to obtain the absorption and emission spectra of components separated on thin layer chromatograms. He described the development of the instrumental parameters and problems of measurement. In a comparison of five different methods for quantitation of trimethylxanthine and acetylsalicylic acid, the results of the *in situ* quantitation by scanning of thin layer chromatograms seemed superior to the other methods. Stahl and Jork (29) summarized their experiences with the Zeiss thin layer chromatogram scanner in 1968. Shellard reviewed the problems of densitometry to 1968 in *Quantitative Paper and Thin Layer Chromatography* (30).

By 1970, there were available some 15 instruments for *in situ* scanning of thin layer chromatograms for quantitation of the separated substances. The development of the Zeiss chromatogram scanner coincided with the appearance of the Schoeffel spectrodensitometer that provided double beam capabilities.

In spite of the advances rapidly being made in instrumentation, there had been little theoretical basis for the application of *in situ* scanning to quantitation of thin layer chromatograms. The theories that have been developed were based on the Kubelka-Munk function. Stahl and Jork (29) stated that straight calibration curves in reflectance measurements are only obtained when evaluation by this function is carried out. Scattering coefficient and molecular absorptivity are considered in the function. When absorption measurements were carried out , one comment they has was:

> Densitometric evaluation is carried out mainly in transmitted light even when the layers are only translucent, so that unforeseen light scattering in the stationary phase can falsify results. In addition, with this measurement setup the differences in layer thickness have a particularly marked effect. Consequently spectrophotometric reflectance measurements on thin layer chromatograms are much better to assess.

This may have been the beginning of the present controversy over whether transmittance or reflectance measurements are the more advantageous.

Goldman and Goodall (31) made a practical evaluation of the Kubelka-Munk theory using the Joyce-Loebl instrument. They showed on theoretical grounds that measurement by transmission is more advantageous than by reflectance. They derived a relatively simplified expression that reduced the need for empiricism in quantitative analysis on thin layer chromatograms using *in situ* scan-

ning spectrodensitometry. Shortly thereafter, Boulton and Pollak (32, 33) published a series of articles describing their evaluations of thin layer chromatograms by direct scanning, both from the practical as well as the theoretical standpoints. These authors had considerable experience gained from their work in scanning paper chromatograms that was summarized in a review published by Boulton (34).

These workers pointed out that the lower sensitivity level of any scanning device is limited by "noise" of both electrical and optical origins. The "noise" in single beam devices is predominantly optical in origin. In double beam instruments, two light beams are used to provide sample and reference signals. By subtracting the "reference" signal from the "sample" signal, a marked increase in sensitivity of detection and improvement in background noise is obtained. The ratio between the two signals has also been used.

Most sorbents used in chromatography have, besides absorbance, a relatively high coefficient of scattering. Theoretical expressions describing the optical transfer in this type of media are rather complex. The simplified versions developed by the two groups are adequate. If the concentration of the solute in the sorbent is not too high, it can be assumed that it affects only the absorbance, while the coefficient of scattering remains unchanged. If the sorbent is nonscattering, the exponential dependence of transmittance in concentration postulated by Beer's law holds. Reflectance measurements do not give information about the interior of the layer. A layer with no scatter is not suitable for reflectance measurements. As scatter increases, deviation from Beer's law increases. The slope of the curve of transmittance versus absorbance decreases with increasing scatter, resulting in a loss of sensitivity and accuracy. For small concentrations, deviations are not enough to be of consequence.

Some practical aspects of the above theories were presented by Touchstone et al. (35). The instrument used was the Schoeffel spectrodensitometer having a double-beam mode and an analog converter to convert the ratio of the two signals to optical density units that appeared as peaked curves on a recorder when a chromatogram was scanned. The utility of the double beam capabilities was illustrated in this report. Again in this work, it was found that transmittance has advantages over reflectance. This appeared to be so whether absorbance or fluorescence determinations were being carried out. A comprehensive treatment of the Kubelka-Munk theories, as applied to quantitative thin layer chromatography, appears in Chapter 2.

A considerable body of literature describing methodology and instrumental parameters for determination of a wide variety of