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Quantitative Thin-Layer Chromatography

A Practical Survey

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ISBN 978-3-642-10727-6 e-ISBN 978-3-642-10729-0
DOI 10.1007/978-3-642-10729-0
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2010938374

Cover design: WMXDesign GmbH, Heidelberg, Germany

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Thin-layer chromatography (TLC) is a rather ignored quantification technique. The method is widely used for education purposes and qualitative analysis. The analysis of herbs, in particular, is often performed by TLC due to the simultaneous separation of different samples facilitating their differentiation at first glance. The aesthetic results of individual coloured TLC zones are certainly appreciated by many people who enjoy looking at such results. Another critical aspect of TLC is the humidity dependence of adsorption chromatography. This is certainly the major reason why adsorption TLC was labelled "irreproducible" and "unreliable" and why industry prefers closed systems such as HPLC. Another reason for avoiding TLC is that analyses using highly automated HPLC were generally superior to TLC, which relies more on the skill of the analyst. Last, but not least, the odour of vanillin reagent reminded laboratory staff on a daily basis that chemical analysis is a part of chemistry and not computer science.

All these aspects that spoke against TLC are now just history. Modern equipment and working practices have overcome all these problems. TLC calibration curves are now linear over more than three orders of magnitude. Modern sample application, development chambers, and reagent spray or dipping devices provide the required degree of automation, reliability, and independence of the local environment (temperature, humidity, etc.) associated with robust analytical methods. Nevertheless, quantitative TLC, unlike HPLC, is still mostly done in resource-limited laboratories with incomplete instrument support.

This book is written as a self-study guide for professional scientists to refresh their understanding of modern TLC. It presents the complete theory of quantitative TLC analysis. It is also written for newcomers who want to use quantitative TLC but have limited access to older books which are often unavailable or difficult to obtain. The main concept was to collect in one place all the knowledge necessary to perform quantitative TLC. The chapters follow a modular style facilitating access to information relevant to the individual operations of a successful TLC analysis. The book starts with a chapter on history followed by a chapter on theory (including practical hints for fast and reliable method development). Chapter 3 introduces the different stationary phases and Chap. 4 the various mobile phases. Chapter 5

describes sample pre-treatment techniques and methods for sample application. Chapter 6 deals with the different development techniques. Chapter 7 adopts a dictionary-like style to introduce the many methods of general and specific staining reactions for visualization. Chapter 8 concentrates on analysis linked to bio-effective methods bridging the gap with biology. Chapter 9 describes all known measurement methods for evaluating TLC separations. Chapters 10 and 11 contain the theory for spectrometric methods linked to Chap. 12 that deals with chemometric methods of data analysis to maximize the information contained in the measured data. The book ends with two chapters (Chaps. 13 and 14) on basic statistics and planning and validation of TLC analyses.

Our hope is that this book will demonstrate that quantitative planar chromatography is a practical alternative in liquid/solid and liquid /liquid separations. We hope that the liquid/solid separation method, which is TLC's strength, will thus find new friends. In 1987 Friedrich Geiss wrote in the preface of his book *Fundamentals of Thin Layer Chromatography* "TLC is here to stay". We believe indeed that TLC will consolidate its position in analytical chemistry. Taking the biological measurement techniques of Chap. 8 into account, we hope that TLC will even extend its range and position among separation methods.

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October 2010

Acknowledgements

From my time in the industry, I personally remember the delighted expressions of laboratory staff looking at a nicely stained TLC plate. I remember in the mid-1980s that TLC was widely used for quantification purposes. I also remember that my technicians were always suspicious of TLC due to its non-linear calibration curves. It was difficult for them to accept that a doubling of the amount of substance did not result in a doubling of the measurement signal. All this was the reason for me to write a TLC book that helps to overcome this problem. I am indebted to the ladies of the Offenburg library who nearly instantly provided the desired papers for this book. I wish to thank Trisha Cornforth for her help in interpreting my German text into English, and I wish to thank my wife Marion and my daughter Johanna for their understanding and patience.

Offenburg
October 2010

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Contents

Chapter 1
History of Planar Chromatography

Generally speaking, spectroscopy and spectrometric methods such as infra-red (IR) spectroscopy and ultra-violet (UV) spectrophotometry are incapable of carrying out complete analysis of complicated mixtures. As a consequence, the individual substances must be separated from one another before spectrophotometric determination can take place.

Chromatography is the term used to describe all separation methods, based on the distribution of compounds between two separate phases. In thin-layer chromatography (TLC), one phase is fixed on a plate (stationary phase) and the other phase is mobile and migrates through the stationary phase (mobile phase). During the chromatographic development process, the mixture to be separated is distributed between the stationary and mobile phases.

Paul Karrer had been involved in developing new identification methods for two decades before he spoke about them at the 1947 IUPAC Congress. During his main lecture he declared "... no other discovery has exerted as great an influence and widened the field of organic chemistry investigation as much as Michail Semenovich Tswett's chromatographic adsorption analysis" [1].

Indeed, modern analysis would be unthinkable without column and planar chromatography. In the long run, modern developments such as the reversed-phase (RP) technique or high-performance thin-layer chromatography (HPTLC) ensure that TLC is frequently used as a separation process [2].

1.1 History of Paper Chromatography (PC)

Writing his book *Libri naturalis historiae* in ancient Rome, Caius Plinius (Pliny the Elder) mentioned using papyrus impregnated with gall-apple extract to identify the addition of iron sulphate in verdigris (copper acetate) [1]. Plinius used papyrus to hold chemical substances where various reagents were mixed and brought to a reaction due to capillary flow in the papyrus. Thus papyrus was used to carry out

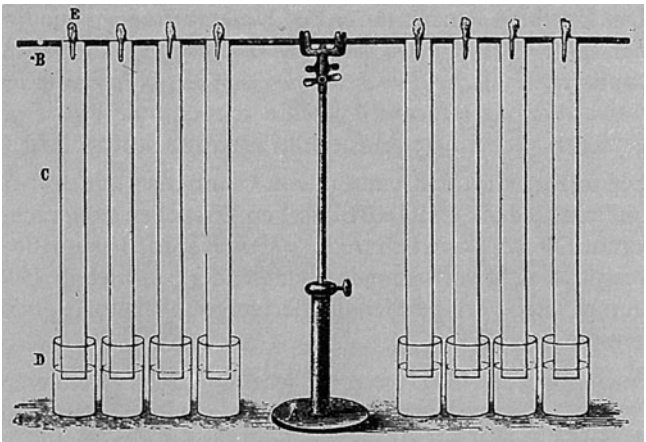
B. Spangenberg et al., *Quantitative Thin-Layer Chromatography*,
DOI 10.1007/978-3-642-10729-0_1,

Fig. 1.1 Capillary picture by Runge

analysis but no substances were separated. The true origins of modern chromatography are of a more recent date. In 1850, Runge (1794–1867) first used paper to differentiate between colour dyes. Runge was the first to use "the power of the hairs in paper" to separate individual substances out of a mixture (Fig. 1.1). He wrote: "Due to the power of its hollow hairs, it (the paper) was capable of separating drops of liquid into component parts according to their individual fluidity, to form a picture with a dark centre and paler outer circles or completely non-coloured rings" [3].

Runge's book (German title *Bildungstrieb der Stoffe*) became famous for its colourful pictures and descriptions as active examples of the "living power in plants and animals". At the World Exhibition held in Paris in 1855, he was awarded a special medal for his book [4]. Like Plinius' paper analysis, Runge used various mixtures to see how they would react on paper [5]. Runge was the first person to deliberately use the characteristics of paper to separate substances, although similar work had been carried out during the same decade by Schönbein (1799–1868). The latter published his experiments in 1861, describing how he had cut unglued paper into strips and dipped them in dissolved dyes [5].

He noticed that the water from the dissolved dyes always moved through the fabric quicker than the dyes themselves and that various dyes moved at different speeds. Goppelsröder (1837–1919) later extended these experiments to kieselguhr and wood fibres. In 1906, a summary of his work concluded that paper manufactured by the company "Schleicher und Schüll", in Düren, Germany, showed the best results [6]. However, as he himself wrote in 1910 "Naturally such dyes cannot be



Ich hänge die Streifen *C*, wie sich aus der Figur ergiebt, mit ihrem unteren Ende 5 bis 10 mm in die zu untersuchenden Lösungen *D* eingetaucht, an einem Glasstabe *B* auf, an welchem sie mit Hilfe jener hölzernen Klammern *E* festgehalten werden, wie sie zum Aufhängen der Wäsche in den Haushaltungen dienen. Ich lasse die Streifen je nach den Lösungsmitteln 15 Minuten bis ein oder mehrere Stunden, selbst bis 12 Stunden hängen. Nachher hebe ich sie aus den Flüssigkeiten heraus

Fig. 1.2 Separation on paper strips according to *Goppelsröder* [7]

completely separated from one another during the first capillary attempt" because "the lower layers still contain small residual amounts of those dyes which have mostly moved upward" [7, 8].

The deficiency of all the so-called frontal analysis separation publications up until then was that the solvent used for the sample and the mobile phase were identical. As Goppelsröder correctly observed, that meant each new separation needed a new sample solution, thus continually contaminating separated substance zones (Fig. 1.2).

Quite independently of Goppelsröder, Reed tried separating many dissolved salts by "*selective absorption in bibulous paper*". It is historically interesting to note that his article published in 1893 finishes with the statement: "I have obtained satisfactory results ... by using tubes containing powdered kaolin that had been slightly pressed down, on which the solution was placed and allowed to soak downwards" [9, 10].

It was the brilliant Russian botanist Tswett (1872–1919), born in Italy and educated in Switzerland, who actually discovered chromatography in 1903. He made the distinction between sample, mobile, and stationary phase, as well as the single application of a sample and the permanent effect of the mobile phase. He was the first to make a successful separation of leaf dyes by means of chromatography

columns [11–16]. Tswett used icing sugar, insulin, $CaCO_3$, aluminium oxide, and many other adsorbents as stationary phases. In 1906, he wrote:

> "There is a definite adsorption sequence according to which the substances can displace each other. The following important application is based on this law. When a chlorophyll solution in petrol ether is filtered through the column of an adsorbent (I mainly use calcium carbonate which is tightly packed into a narrow glass tube), then the dyes will be separated according to their adsorption sequence from the top down in various coloured zones, as the more strongly adsorbed dyes displace the more weakly retained. This separation is practically complete if, after passing the dye solution through the adsorbent column, the latter is washed with pure solvent [13]".

This is the first description of placing a sample on the stationary phase, the effect of the (clean!) mobile phase on the sample, as well as the creation of substance zones and their broadening in the course of the elution. The genius of Tswett's discovery can be recognized by the fact that Goppelsröder had hung paper strips in hundreds of solutions for more than 40 years without making the obvious conclusion (as seen from today's point of view) that the paper strips should be further developed after immersion in clean solutions.

In the first of his three articles, published in 1906 in *Berichte der Deutschen Botanischen Gesellschaft* (Reports of the German Botanical Society) [13, 14], Tswett first mentioned the words "chromatogram" and "chromatography", both concepts he had devised himself (Fig. 1.3). He wrote:

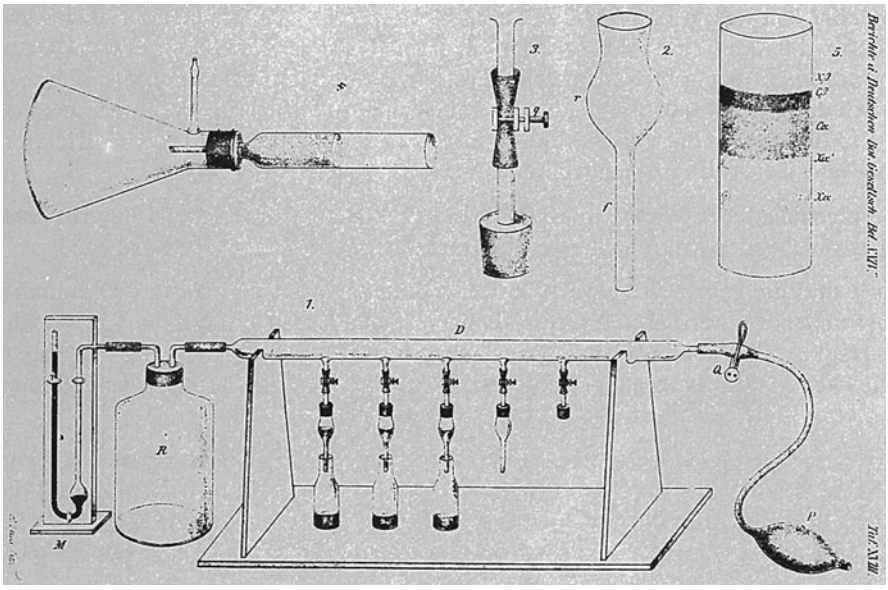


Fig. 1.3 Tswett's apparatus for column chromatography [14]. The small columns were activated by slight over-pressure. Shows chromatography carried out under pressure

> "Like light rays in the spectrum, the different components of a dye mixture, obeying a law, are separated on the calcium carbonate column and can thus be qualitatively and quantitatively determined. I call such a preparation a chromatogram and the corresponding method the chromatographic method [13]".

Tswett associated the concept of "chromatography" with the literal translation of "colour description" and was convinced he had discovered a universally applicable method. He wrote [13]:

> "Of course, the described adsorption phenomena are not only confined to chlorophyll dyes. It is to be assumed that all kinds of coloured or colourless chemical compounds are subject to the same laws. So far I have successfully examined lecithin, alkannin, prodigiosin, sudan, cyanin, solanorubin as well as acid derivates of chlorophylline".

Tswett listed over 100 substances he had tested for their adsorption capacities [13]. He definitely carried out his first experiments with filter paper although nowadays we cannot be sure whether Tswett really invented paper chromatography. However, the invention of chromatography as a separation method using filled columns can definitely be attributed to him, and he even recognized the method's full potential. In 1906, he wrote:

> "Now the question can be proposed whether the chromatographic method can be raised to a chromatometric power. It would be desirable to express the quantities of dyes simply in volumes of adsorbent saturated by them. The experiments which I have performed to this end have thus far led to no satisfactory results [14]".

A further step on the way to modern TLC was the discovery of circular paper chromatography by Grüss in about 1908. Following on work by Goppelsröder, and in a similar way to Runge, Grüss dropped samples (mainly enzymes) onto the centre of a round filter paper. After the formation of rings, he added water to the centre of the paper, and a second water application caused complete separation of the substances. Grüss used colour-creating reagents to make colourless zones visible, using the name "chromogram" for the "rows of coloured sections lined up beside one another" [17].

Schönbein and Göppelsröder's work on "capillary analysis" was continued by Liesegang who went way ahead of his predecessors with a remarkable piece of work on 15 May 1943. He described his work under the title *Cross-Capillary Analysis*:

> "A drop of a dye mix was left to dry on the corner of a sheet of filter paper about 20 × 20 cm in size. When water was allowed to soak through by a capillary process, the result was a narrow strip of colour. In contrast to normal capillary analysis, separating into individual colour strips is not usually as sharp as can be achieved by chromatographic analysis by subsequent development. Further separation can be achieved by drying the paper and again hanging it in the water so it rises perpendicularly to the first capillary direction. Moreover, the kind of polymerisation of individual dyes can thus be spatially separated by observing their mobility rate. The capillary rise of water can be replaced by organic fluids and the filter paper replaced by a gypsum plate, etc." [18]".

Because the Second World War was raging at the time, this extremely important advance in paper chromatography remained unrecognized and did not influence later researchers [19].

In 1930, Willstädter gave the only German translation of a Tswett dissertation (dated 1910) to Richard Kuhn (1900–1967). This led to a renaissance of Tswett's chromatographic methods in the 1930s. In 1938, Kuhn was awarded the Nobel Prize for his work on column chromatographic separations of carotenoids. His assistant Winterstein played an important role in disseminating information about "column adsorption chromatography" through numerous lectures and experimental demonstrations. This insured that the breakthrough in column adsorption chromatography was not lost this time around. It was also a 1933 Winterstein lecture at Cambridge that inspired Martin to carry out his first basic experiments in chromatography [1].

In the 1930s, Martin constructed large apparatuses to achieve "against the current extraction" to thus separate substances with similar chemical characteristics. Together with Synge, he explored this method of alternately shaking an extraction medium to separate amino acids. Much effort went into designing apparatuses to facilitate rapid equilibrium of both fluids. In retrospect, Martin wrote in 1975 [1]:

> "I suddenly realized it was not necessary to move both liquids because the required conditions were fulfilled if I just moved one of them . . . Synge and I took silica gel intended as a drying agent from a balance case, ground it up, sifted it and added water . . . We put this mixture of silica gel and water into a column . . . One foot of in this apparatus' tubing could do substantially better separations than all the machinery we had constructed until then".

Martin and Synge received the 1952 Nobel Prize for chemistry for their discovery of distribution chromatography.

Two years after his fundamental recognition of distribution chromatography, 1944, together with Consden and Gordon, Martin published a further variation of the chromatographic separation process. He named this method "a Partition Chromatographic Method Using Paper" [20]. The authors published an exact description of their simple apparatus:

> "a strip of filter paper with the substances applied, with the upper end dipped in a trough filled with water-saturated medium. The filter paper was folded over the upper edge so that the solution could not be soaked up by capillarity. The whole experiment took place in the vapour-saturated atmosphere of a closed chamber".

This type of chromatography revolutionized chemical analysis, significantly facilitating the identification of amino acids derived from proteins. Previously, it required several years to analyse a protein, but this simple separation method shortened the work to a few days! By 1954 more than 4,000 publications had already been published on the subject of paper chromatography. As a contemporary scientist laconically remarked: "Paper chromatography is so widely used that it is impossible to make more than a rough estimate of its applications" [21].

1.2 History of Thin-Layer Chromatography

In 1889, Beyerinck published a separation of substances by diffusion in gelatine smeared on a glass plate [22]. However, this was a first attempt without further consequences to carry out planar chromatography with a stationary phase other than paper.

In 1938, encouraged by Tswett's work, Izmailov (1907–1961) and Shraiber (1904–1992) transferred the results of column chromatography to the so-called open columns. These researchers spread thin layers (about 2 mm) of various sorbents such as lime, magnesium oxide, or aluminium oxide on glass plates [23–26] and performed separations similar to those of Grüss.

In 1992, Shraiber described the discovery of TLC as follows:

> "The use of column chromatography for the analysis of pharmaceutical samples took a lot of time. This limited the application of the method throughout pharmaceutical analysis. For this reason our thoughts and efforts were directed to studying opportunities for accelerating the separation process for complex samples [24, 25]".

Furthermore, *Shraiber* stated that the original idea for planar chromatography derived from Tswett. She wrote:

> "Thin layer chromatographic adsorption analysis was elaborated as a result of a number of experiments based on the separation of a mixture of compounds into zones on a thin layer of adsorbent using one drop of sample. Developing M. S. Tswett's idea, it was demonstrated that the planar adsorbent layer is an analog of the chromatographic column [24–27]".

In 1949, Meinhardt and Hall introduced a significant technical improvement to "surface chromatography". They fixed the aluminium oxide sorbent onto a glass plate with starch as a binding agent [28]. This resulted in stable plates without splits. Kirchner Miller and Keller modified the method according to Meinhardt and Hall. They added the sorbent's zinc silicate and zinc cadmium sulphide as indicators to visualize UV absorbing compounds. Thus substances could be observed on the plate without dyeing or destroying them, under short-wavelength UV light (254 nm) as zones of reduced fluorescence. They named their coated glass strips "chromato strips" and used them for the separation of terpenes. They wrote: "Very unreactive compounds can be located by spraying with concentrated sulfuric–nitric acid mixture and heating to cause charring of the compounds". They came to the following conclusion: "Of the numerous adsorbents tested, silicic acid proved to be the best for terpenes" [29].

In 1954, Reitsema used glass plates (size 12.5 cm × 17.5 cm) coated with silica gel, naming them "chromato-plates". He achieved a high sample throughput by simultaneously applying and developing several samples [30].

TLC's essential breakthrough, both as an analytical separation method and in establishing its name, was the achievement of Stahl (1924–1986). From 1955 onwards he definitively standardized the separation technique and introduced it into routine analysis [31]. Stahl used 20 × 20 cm glass plates covered with various sorbents. He published his manual in 1962 and Randerath's book was dated the

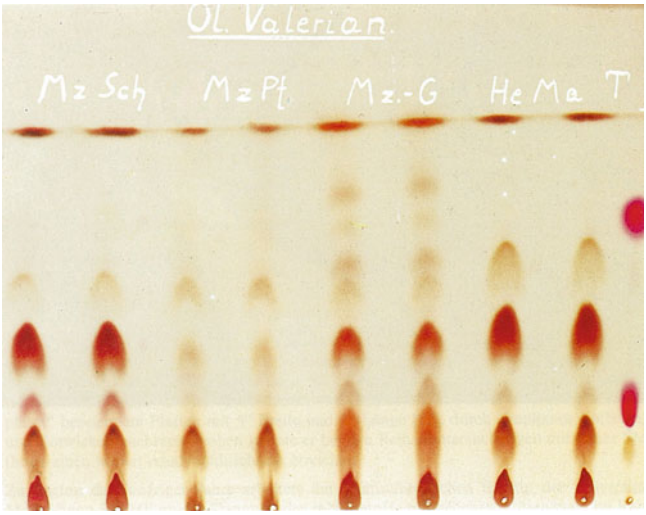


Fig. 1.4 One of the first TLC plates developed by Stahl in 1956 [1], showing a valerian oil separation. The plate was sprayed with $SbCl_3$ solution in $CHCl_3$ and subsequently heated

same year; both made the method world famous under the abbreviation "TLC" (Fig. 1.4) [32, 33].

The next improvements were made at the beginning of the 1970s by Kaiser at the Institute of Chromatography in Bad Dürkheim (Germany) [34]. He prepared silica gel plates without a binder using silica particles of a small size (5 µm) with a narrow size distribution. The separation time was decisively reduced from hours to minutes, while simultaneously improving resolution [34]. This led to the 1977 publication of Kaiser's book *HPTLC – High Performance Thin Layer Chromatography*, which facilitated the adoption of this improved technique [35].

In the middle of the 1970s, Halpaap at Merck (Darmstadt, Germany) invented the so-called high performance thin-layer chromatography (HPTLC) silica gel plates in 10×10 cm format and launched them on the market [36]. The RP-18-HPTLC plate arrived in 1980, followed by Jost and Hauck's amino plate in 1982, the Cyano HPTLC plate in 1985 and the Diol HPTLC plate in 1987 [37].

1.3 The History of Quantitative Planar Chromatography

In 1953, Cramer indicated various possibilities for obtaining quantitative results by means of paper chromatograms [38]. In 1954, a Merck company brochure noted:

> "There is a proportional relationship between the size of the spot and the logarithm of the substance concentration. Therefore, if equal spot sizes of two solutions of the same substance are "chromatographed" and also result in spots of equal size, both solutions must contain equal concentrations (within an accuracy span of about 10%). The spot size

> can also be determined in this way, so that solutions of various (known) concentrations can be chromatographed and compared with spots of an unknown solution concentration. The spot sizes can either be determined by measuring the surface area or by cutting out the spots and weighing them. Sports can also be evaluated by photometric means. The paper is made transparent by soaking it for five minutes in a mixture with equal parts of α-bromonaphthalene and liquid paraffin (DAB 6). After drying, the spots can be measured with the aid of a photo-electric detector" [39]".

It was also common practice to cut out zones from the layer and determine the amount of substance present by extraction with a micro-soxhlet apparatus as well as by a subsequent colorimetric concentration determination. In the early days of TLC, it was only possible to determine the contents by scratching off the spot and extracting the sample. Despite this primitive quantification method, Kirchner obtained an amazing average error of only 2.8% in 1954 [40].

In 1960, Hefendehl published a method for measuring thin-layer chromatograms without destroying them based on the transmission of light by the layer. He sprayed the thin-layer plate with a paraffin–ether solution (1+1, V/V) to make the plate transparent and then evaluated it by photographic means [41].

In 1963, Barrett and Dallas used TLC plates treated with sulphuric acid for charring. They used a chromo-scan densitometer and also recorded densitograms in the reflectance mode [42, 43].

In 1964, Jork (1933–1993) published the first densitogram measured in the reflectance mode from a stained TLC plate. He used a chromo-scan densitometer produced by the company *Joyce & Loeble* (Newcastle, England). By automatic integration of the peak areas, he obtained good reproducibility with errors less than 1% [43, 44].

After about 1964, quantitative TLC by spectrophotometry in the reflectance mode based on Jork's work became the accepted measurement method. Jork illuminated untreated TLC plates with monochromatic light and recorded the intensity of the light reflected by the plate, depending on its position and wavelength [43].

If a ray of light illuminates the TLC layer, each individual layer particle absorbs light. However, most of the light is scattered and reflected by the plate. This reflected light permits rapid and, most importantly, non-destructive quantification of the substance zones on the plate. The Zeiss KM2 spectrophotometer (that was adapted by Jork as KM3) was later capable of routinely measuring densitograms as well as spectra in the reflectance, transmittance, and fluorescence modes (Fig. 1.5) [45, 46].

The first successful quantitative measurements were carried out with a digital camera by Prosek and Kaiser in 1984 [36, 47]. At the beginning of the 1970s, Ebel had combined a TLC scanner capable of measuring light in the reflectance and transmittance modes with a desktop computer to control the plate photography, which was thus capable of evaluating the received reflectance data [48].

The first spectra of a light fibre TLC scanner in the range of 300–700 nm were published by Hamman and Martin [49]. The first combination of a TLC scanner with a diode-array detector (DAD detector) was published by Bayerbach and

Fig. 1.5 The first TLC scanner (ZR3), constructed by Jork for Zeiss, Germany [45]

Gauglitz in 1989 [50]. In 1998, J&M Company (Aalen, Germany) brought out the first diode-array light scanner for HPTLC plates [51]. For a complete overview of more than 50 years of TLC publications and instrumentation development, see [51].

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Chapter 2
Theoretical Basis of Thin Layer Chromatography (TLC)

2.1 Planar and Column Chromatography

In column chromatography a defined sample amount is injected into a flowing mobile phase. The mix of sample and mobile phase then migrates through the column. If the separation conditions are arranged such that the migration rate of the sample components is different then a separation is obtained. Often a target compound (analyte) has to be separated from all other compounds present in the sample, in which case it is merely sufficient to choose conditions where the analyte migration rate is different from all other compounds. In a properly selected system, all the compounds will leave the column one after the other and then move through the detector. Their signals, therefore, are registered in sequential order as a chromatogram. Column chromatographic methods always work in sequence. When the sample is injected, chromatographic separation occurs and is measured. This type of chromatography is known as "Online Chromatography".

The different column chromatographic methods can be distinguished by their phase systems. Gas chromatography uses an inert gas such as nitrogen or helium as the mobile phase. In liquid chromatography the mobile phase is a liquid with a constant or varied composition altered during the separation process. A separation employing a constant mobile phase composition is known as an isocratic separation. If the mobile phase composition is varied during the separation this is called a *gradient* separation. A pump is used to move the mobile phase through the column at a suitable velocity. Separations are optimized by first selecting a suitable column and then varying the mobile phase composition to achieve the desired resolution in an acceptable time.

For planar separations like TLC, different samples are usually applied to the stationary phase before it is contacted by the mobile phase which begins to migrate through it in a definite direction. The movement of the mobile phase through the stationary phase is referred to as the development step. After development the mobile phase is removed by evaporation and detection is performed in the

B. Spangenberg et al., *Quantitative Thin-Layer Chromatography*,
DOI 10.1007/978-3-642-10729-0_2,

stationary phase. The record of the detector response plotted against the separation distance is called a densitogram.

Separations by planar chromatography occur in parallel in contrast to the sequential approach of column chromatography. This situation has advantages and disadvantages: a sequential process like column chromatography facilitates automation in which a fixed protocol is commonly employed for a batch of samples.

Planar chromatographic separations are more flexible but not easily automated, and the sequence of manual steps commonly used makes validation of the method more difficult and has led, for instance, to the fact that the pharmaceutical industry hardly ever uses planar chromatography to check medicinal products (Fig. 2.1).

Another important difference between planar chromatography and column chromatography lies in a more flexible use of the stationary phase. A new stationary phase is needed for each separation in planar chromatography, thus preventing any cross-contamination from one sample to another. Thus even heavily contaminated samples can be applied to the stationary phase without sample cleanup. Sample components are not usually overlooked during detection because the whole separation can be scanned. Column chromatography only measures those substances that leave the

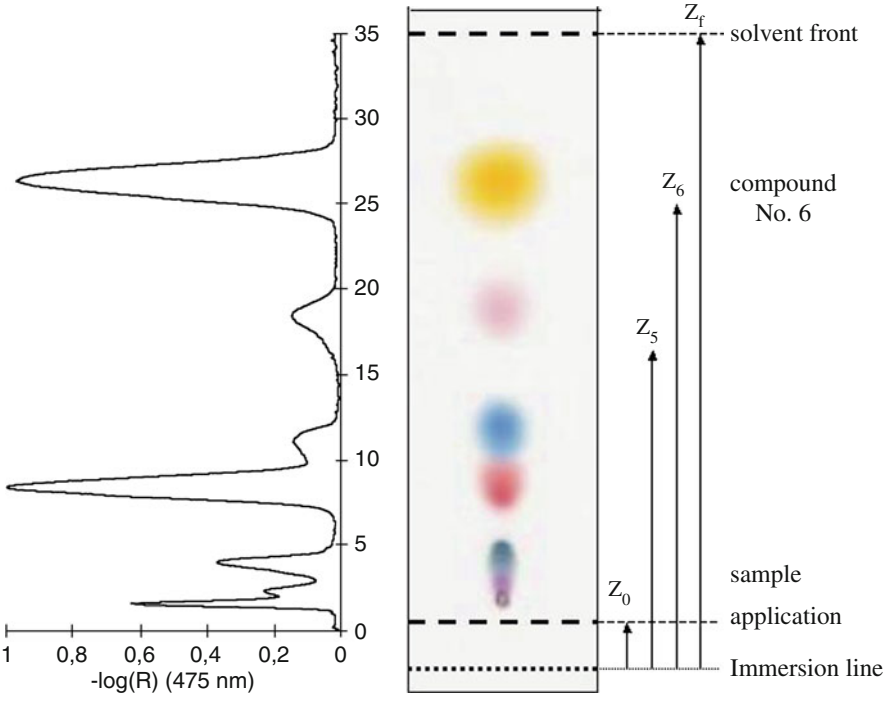

Fig. 2.1 Separation of six dyes (CAMAG III mix, no. 032.8003), with the relevant densitogram, on a SiO_2 plate, developed with toluene. With increasing R_f values: Ciba F-II (*violet*), indophenol (*indigo blue*), ariabel red (*red*), sudan blue II (*blue*), sudan IV (*scarlet red*) and (6) *N*,*N*-dimethylaminobenzene (*yellow*)