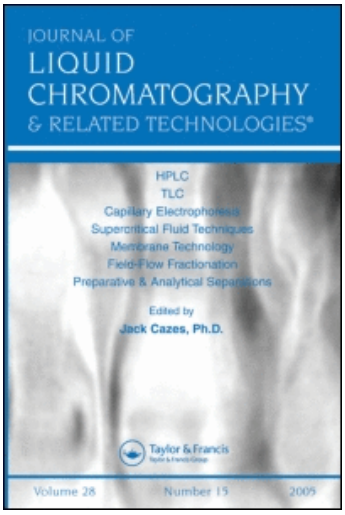

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Modern Thin-Layer Chromatography: Advances and Perspectives

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MODERN THIN-LAYER CHROMATOGRAPHY: ADVANCES AND PERSPECTIVES

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ABSTRACT

A review of the recent advances in TLC stationary phases, sample application, developing and quantification devices is presented. Also, a comparison between the properties of TLC and HPTLC is given. The use of modern instrumentation for multiwavelength scanning is discussed.

INTRODUCTION

The last decade have witnessed a phenomenal advance in all aspects of thin layer chromatography (TLC), high quality high performance, multimodal TLC plates (supports), accurate and precise spotting techniques, instrumentalized development devices and sophisticated densitometers. These advances make TLC a modern instrumental technique which is amiable to automation and computerization which seems to be a must in today's modern analytical laboratory.

This article will review the most prominent TLC advances with special emphasis on quantitation and instrumentation and less on application. The objective is to introduce the reader, and to familiarize him with the advances in TLC since the introduction of high performance TLC (HPTLC) plates, which have not only several advantages but also requirements over regular TLC plates. These requirements lead to the development of new apparatus and instruments for accurate and reproducible quantitative TLC (QTLC).

HISTORY, ACCEPTANCE AND GENERAL ASPECTS OF TLC

Thin-Layer Chromatography was described and routinely used by Kirchner and his group since 1951 (1). Five years later Stahl (2) introduced standardized equipment and techniques, and the method found rapid acceptance for qualitative analysis. Serious efforts to extend TLC into a quantitative method began in the 1960's, when several TLC-dedicated densitometers appeared on the market and appreciable results were obtained.

As high performance liquid chromatography (HPLC) became established during the 1970's, interest in QTLC declined. This is due to the fact that HPLC offered the better chromatographic resolution, in most cases the better precision of quantitative results, and, as an on-line process, better conditions for automation. To most analysts, HPLC appeared to be a superior method that could completely supersede QTLC.

In recent years, QTLC has regained interest as the number of publications show (3). More and more analysts accept the fact that both HPLC and QTLC have their specific merits and often complement one another. Some typical features that can make QTLC the method of choice for a given analytical problem are listed in a concise form in Table 1.

As mentioned earlier, to find acceptance in modern analytical laboratories TLC had to become an instrumental technique, which other chromatographic techniques like GC and HPLC have always been.

Table 1. Aspects that can make instrumental TLC the method of choice for a given analytical problem.

TLC FEATURES	...BASED ON
Simplified sample preparation	stationary phase disposable
Large sample volumes ...	sample spray-on technique
... low detection limits	
Many samples simultaneously	"off-line" principle
In-situ derivatization	"off-line" principle, storage principle
Mobile phase immaterial for detection	detection in absence of the mobile phase
Quantification/identification can be repeated	storage principle, plate is data base
Extreme flexibility	"off-line" principle

Before discussing the different aspects of TLC, let us review the requirments of QTLC, the advantages and the limitations.

a) Chromatographic separation efficiency of HPLC is usually better than that of TLC, due to the limited separation distance of the latter. N values of 10.000-20.000 in HPLC as compared with 5.000-10.000 in HPTLC is state of the art (5). Improvements can be expected in both techniques so that the relation will be maintained more or less.

b) In general, quantitative precision (not necessarily accuracy) in HPLC is somewhat better than in QTLC, although densitometry with structure subtraction (6), will be discussed later, has been reported to bring QTLC to about the same level.

c) 24-hour automation is only possible with HPLC, not QTLC. It should not be overlooked, however, that the number of analyses that can be handled by one QTLC system per working day, exceeds that of a fully automated HPLC system operated 24 hours.

To conclude from these considerations: Although HPLC, for good reasons, is the more often employed method, QTLC provides its own set of distinct advantages which could not be exploited until the recent introduction of modern instrumentation for TLC. Therefore, a combination of both techniques provides the modern analytical laboratory with a maximum of flexibility, economy and reliability of results.

Regarding automation, one should bear in mind that QTLC analysis is a typical off-line or open process, and many of its merits derive from this fact. Its "vertical automation", i.e. the attempt to include the individual steps in a fixed sequential procedure, as has been suggested from time to time (7), would eliminate several of the advantages, not to speak of flexibility. Therefore, automation of the individual steps of the TLC procedure appears more appropriate, and is already available.

HPTLC PROPERTIES

The development of conventional TLC into a modern instrumental technique started with the introduction of the so called high-resolution (HPTLC) materials in 1975. The expression HPTLC refers to layers which have a better separation efficiency over a shorter separation distance (50 mm instead of 100-120 mm) due to smaller particle size, 5 µm vs. 12 µm and particularly a more uniform particle size. Table 2 summarizes the properties of both TLC and HPTLC. The speed of migration of a liquid flowing in a TLC layer by capillary action decreases with the square of the distance. Accordingly, on HPTLC layers, due to their shorter optimum separation distance, chromatography takes place on HPTLC layers, chromatog-

Table 2. Properties of TLC and HPTLC

	TLC	HPTLC
Plate size (cm)	20 x 20	10 x 10 or 20 x 10
Particle size (μm)	11 to 12	5
Layer thickness (mm)	0.25	0.20
Sample volume (μl)	1 to 5	0.1 to 0.5
Spot diameter (mm) before development	3 to 6	1 to 1.5
Spot diameter (mm) after development	6 to 12	2 to 5
Optimum Solvent migration distance (cm)	10 to 16	3 to 6
Developing time (min.)	20 to 180	3 to 20
Detection limits (ng)		
Absorption	50 to 5,000	5 to 500
Fluorescence	1 to 1,000	0.1 to 100

raphy takes place in the fast capillary flow range of the respective developing solvent. This accounts for practically all advantages of HPTLC over conventional TLC (4). These include: (a) faster separation, hence reduced diffusion, i.e. better separation efficiency; (b) detection (determination) limits reduced by a factor of 10-15; (c) better economy: lower plate costs per sample, less solvent comsumption.

To utilize the advantages of HPTLC stationary phases it became necessary to: (a) reduce the spotted sample volumes appropriately, i.e. one tenth of sample volumes applied in conventional TLC; (b) use high quality sample application, i.e. the smallest possible spot size, and to position the samples with great precision; (c) use developing techniques that make

the chromatographic process reproducible; and (d) employ highly precise and reliable densitometric evaluation procedures.

All these requirements called for the development and consequent application of appropriate TLC instrumentation. However, before discussing today's HPTLC equipment, a discussion of available TLC supports is in order.

SINGLE AND MULTIMODAL TLC PLATES

Characterized by their properties, stationary phases for HPTLC are known as normal (silica gel), reversed-phase (alkyl- or phenyl-substituted), ion-exchange, or chiral plates (for optically active isomeric mixtures). Cellulose, polyamide, and alumina also are used.

Reversed-phase C_{18} columns are the most popular ones in HPLC, while in TLC, silica gel is more widely used. Most commercially available TLC plates are coated with a single support. A TLC plate coated side-by-side by C_{18} and silica gel is available. A two phase plate (silanized and silica gel) was used for the separation of a mixture of an oxidation product of cholesterols (8).

Recently, a series of HPTLC plates has been introduced that are coated with supports exhibiting multimodal properties. Unlike alkyl-derivatized silica gel supports, i.e., C_2, C_8, and C_{18} reversed-phase plates, which are of hydrophobic character and are mainly used with aqueous/polar organic mobile phases in a single mode, the amino- and cyano-modified silica gel plates are of hydrophilic character and can be used as a multimodal medium for the separation of hydrophilic or charged substances. This gives the chromatographer a wide choice of selectivity. For examples see Tables 3 and 4. For detailed discussion the reader is referred to the original articles (9, 10).

Available also are chiral plates which are used for the separation of enantiomers based on ligand exchange chromatography. Gunther et al (11, 12) were able to resolve the D and L enantiomers of underivatized amino acids.

Table 3. Separation of an adenosine mixture using an amino-modified silica gel plate and a mobile phase of water, and water/methanol with the addition of 0.18 M NaCl

	Rf	
Compound	Water	30% Water/Methanol
ATP	0.55	0.01
ADP	0.67	0.05
AMP	0.80	0.21
cAMP	0.84	0.64
Adenosine	0.90	0.74

Table 4. Comparison of the separation of a steroid mixture using a cyano-modified silica gel plate and a mobile phase of (90:10) petroleum ether: acetone (A) and (60:40) acetone: water (B)

	Rf	
Compound	Eluent A	Eluent B
Cholesterol	0.80	0.05
Androsterone	0.40	0.40
Cortexolone	0.20	0.45
Corticosterone	0.10	0.55
Cortisone	0.20	0.60

SAMPLE APPLICATION/SAMPLE DOSAGE

It was first shown by Kaiser (13) that as the resolution power of the layer improves, so must the quality of sample application. Apart from precise positioning (essential when densitometric scanning is employed without an automatic spot optimization routine) the quality of sample application increases as the sample originating zone decreases. For HPTLC layers the maximum size (in the direction of chromatography) should not exceed 1.5 mm. This means, the maximum sample volume that can be dispensed from a capillary pipette or from a syringe in one stroke onto an HPTLC layer without compromising application quality is 500 nanoliters.

Sample volumes of 100 and 200 nanoliters can be dosed with the respective nanopipettes, 500 nL with a disposable glass capillary. It is essential that these fixed volume pipettes are precisely guided with respect to the lateral and vertical position, which will simplify densitometric measurements and result in more accurate quantitation. This is possible with currently available mechanized spotting devices, Figures (1,2). As an alternative to a capillary pipette a micrometer controlled syringe for variable volumes can be guided by the same spotting device.

Fully automatic TLC samplers are available with a dynamic range from 100 nl to several microliters with a volume precision better than 1% (or 5 nL respectively). They provide a positional precision of ± 0.05 mm. Since sample dispensing speed is selectable over a wide dynamic range, considerably larger volumes than 500 nL can be applied without sacrificing sample application quality.

A better alternative to sample application in the form of spots is application of the sample as narrow bands. This provides the highest resolution attainable with the relevant TLC system (14). In addition, certain systematic measuring errors liable to occur with densitometric evaluation are reduced by employing the aliquot type scanning technique, i.e. scanning with a slit shorter (1/2 or 1/3) than the band applied.

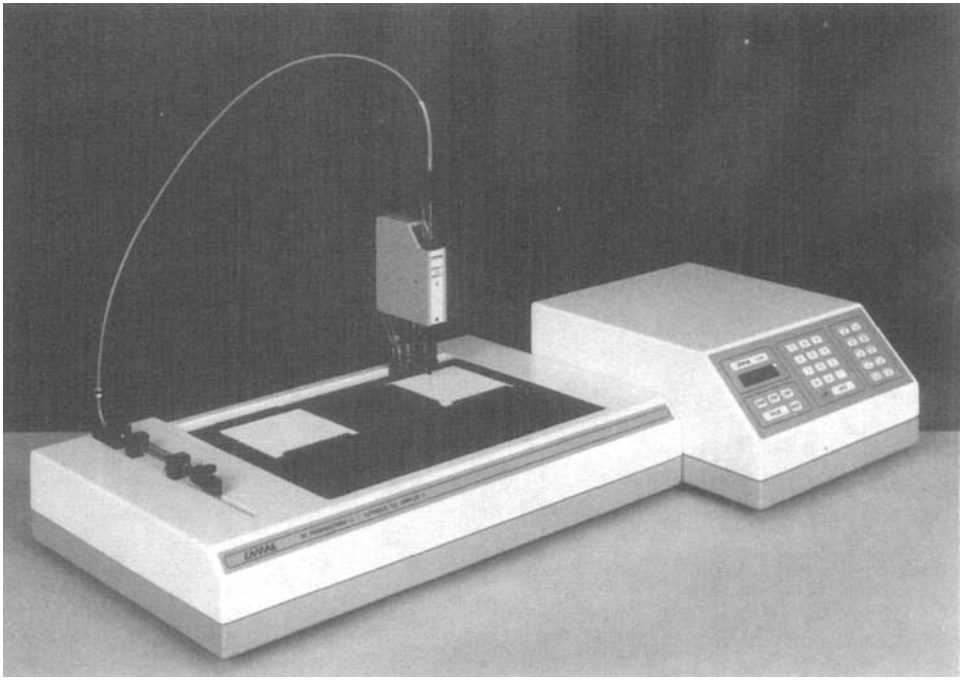

Figure 1. Automatic TLC spotting device.

Current specialized instrumentation allows sample application in narrow bands by a spray-on technique, which combines application with sample concentration. Sample volumes between 2 µl and more than 10 µl can be applied onto HPTLC layers. This technique lowers the detection limit with respect to concentration of the solution. Another benefit of the spray-on technique is the ability to safely apply different volumes of the same solution instead of the same volume of different concentrations, thus greatly reducing the amount of effort required for calibration.

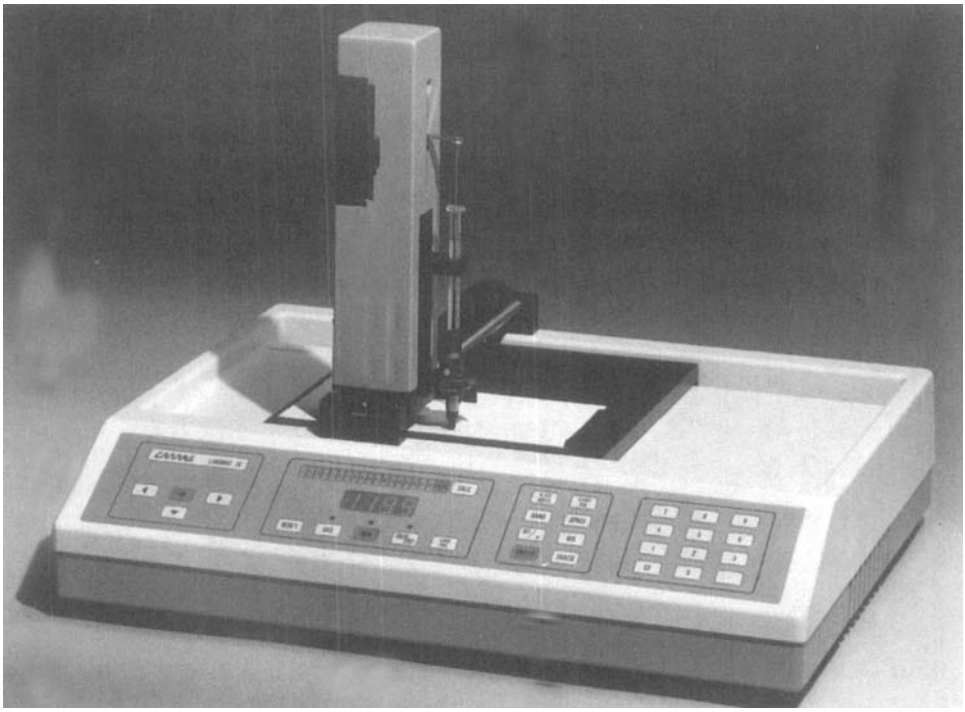

Figure 2. Automatic bandwise sample application using a spray-on technique.

TLC PLATE DEVELOPMENT

After spotting the plate can be developed in the ascending, horizontal, circular and antiradial modes, in an isocratic or gradient mobile phase at room temperature or in the cold with or without pressure. The conventional way to develop a thin-layer chromatogram is to immerse the lower edge of the plate in the developing solvent in a suitable tank. Using a twin trough chamber offers advantages with respect to a better control of chromatographic conditions, e.g. one trough is filled with the developing

while the other with the conditioning solvent. It also requires little more solvent than is required for chromatography. This makes it acceptable to follow the recommendation to always use fresh solvent for every plate, to achieve reproducible results.

Even more economy is provided using the HPTLC linear (horizontal) developing chamber. Here samples are applied parallel to both opposing edges of the plate which is then developed from both sides simultaneously, Figure (3). This way the number of samples per plate can be doubled. It is to be noted, however, that the sandwich configuration maintained in this chamber is not suitable for all developing solvents.

The sandwich configuration is suitable for neat solvents and for solvent mixtures that behave like a one-component solvent. Examples for such solvents are: hexane - acetone (85:15), toluene - dioxane - methanol (8:2:1), chloroform - ethyl acetate (1:1), and ethyl acetate - methanol (95:5).

The sandwich configuration is not suitable for all solvents that contain volatile acids or bases, and for mixtures with a comparatively large concentration of a polar solvent such as methanol and/or acetonitrile.

Unless the sandwich configuration cancels out for these criteria, it is recommendable to always try the HPTLC Linear Developing Chamber for a given separation problem as it offers unsurpassed economy. Plates can also be developed in the circular or anti circular (antiradial) format. The circular gives better resolution while the antiradial allows the spotting and analysis of a larger number of samples on the same size plate.

Automated Multiple Development: A recent concept in plate development is automated multiple development (AMD) which is comparable to gradient elution in HPLC, Figure (4). This new concept has been derived by Burger (15, 16) from a technique called programmed multiple developing process (PMD) described in 1973 by Perry et al. (17). The plate is developed repeatedly

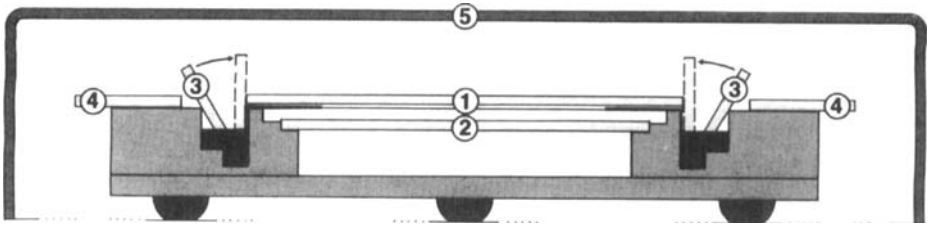

Figure 3. HPTLC linear developing chamber: 1) chromatogram plate, layer facing downward, 2) couter plate, 3) glass strips forming capillary slit when tilted inward, 4) covers over solvent troughs, 5) perspex hood.

Figure 4. Automatic multiple development system.

in the same direction over increasing migration distances. Unlike PMD, in AMD the developing solvent for each successive run differs from the one used before, so that a stepwise gradient is obtained. Between developments, the solvent is completely removed from the developing chamber and the layer is dried under vacuum. The systematic approach of the AMD process provides the ability to separate complex, multicomponent mixtures in a single chromatogram, employing a reproducible elution gradient. Substances widely differing in their polarity can be separated, see Figure (5).

Unlike gradient HPLC, the AMD gradient starts with the most polar solvent (the solvent with the strongest elution power) for which the shortest developing distance is employed, and is varied towards decreasing polarity. The longest migration distance is used with the most non-polar elution solvent. "Universal gradient" is the term for an AMD gradient that starts with a very polar solvent and is varied via the basis solvent of medium polarity to a very non-polar solvent. The central or "basis" solvent, to a certain extent also the non-polar component, determines selectivity. Either dichloromethane or diisopropyl ether is used as basis solvent in over 80% of all AMD applications to date.

Reproducible gradient elution needs a fully automatic instrument. With all parameters properly maintained, and each developing step longer than the previous one, e.g. 3 mm longer running distance, the densitogram of a chromatogram track can be superimposed with a matched scale diagram of the gradient. This permits easy identification of the region of the gradient that caused certain fractions to be resolved. At the same time, it can be determined, which part of the gradient needs to be modified.

A remarkable feature of the AMD method is that the migration distance of the individual component is independent of the sample matrix. This can be verified by superimposing the densitograms of tracks of a chromatogram on which there were separated side by side: a caffeine standard, a sample of untreated coffee, and a sample of untreated human urine from a subject who

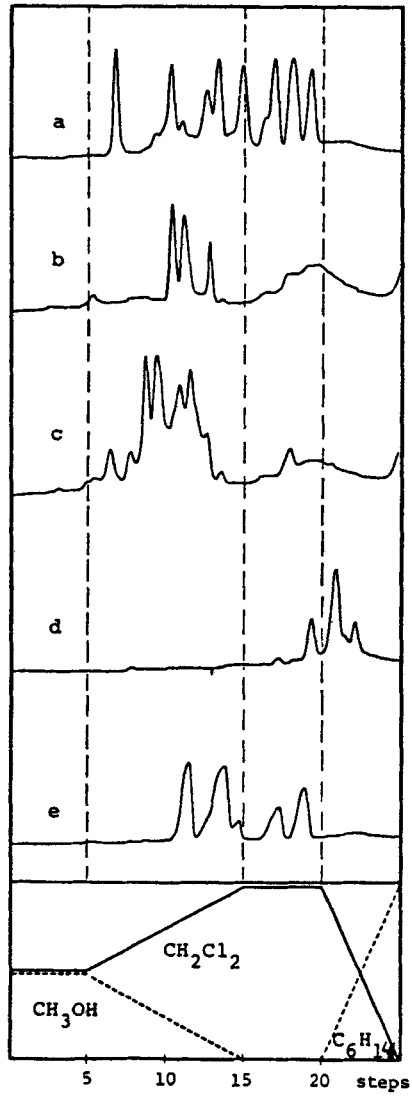

Figure 5. Densitogram curves of AMD chromatogram: a) PTH-amino acids, b) analgesics, c) steroids, d) dyes and e) barbiturates, all chromatographed on same silica gel plate with gradient as depicted.

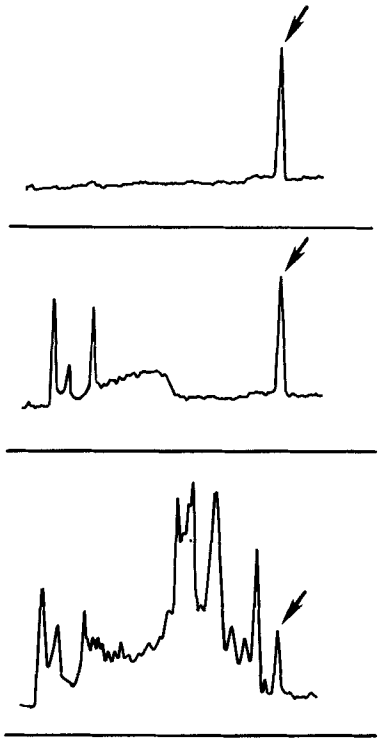

Figure 6. Chromatograms of caffeine standard (top) untreated coffee (middle) and untreated urine from a coffee drinker (bottom).

drank coffee. The caffeine fraction is at the same position on each track, Figure (6).

During the AMD procedure fractions are focused into narrow bands with a typical peak width of about 1 mm. This feature makes AMD an attractive alternative to "overpressure" (forced flow) TLC (OPTLC) (18), the true merit of which is the extension of the useful separation distance of the layer in order to accommodate more fractions. The peak capacity of an HPTLC plate AMD developed is greater than that of OPTLC in spite of the longer separation distance of the latter.

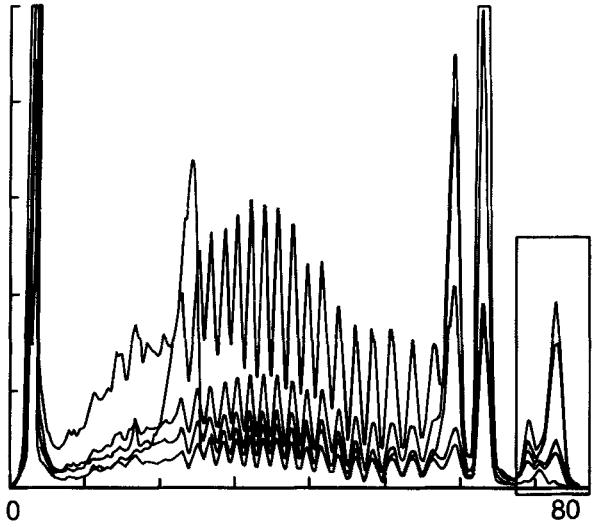

Figure 7. Multiwavelength scan of an AMD separation of a plastic prepolymerizate, upper curve scanned at 200 nm, lowest at 300 nm.

A multiwavelength scan of an AMD separation of a plastic prepolymerization product with additives reveals that a peak capacity of about 80 is achievable by this technique, Figure (7).

Overpressured TLC: This is another approach to instrumentalized plate development (18). The concept is simple, the plate after spotting and drying is placed in the apparatus and covered with an inert see through plate. The solvent is then forced into the plate and causes the resolution of the different components in the mixture. OPTLC in a way immitates HPLC but takes advantage of the large number of samples spotted on the plate and analyzed simultaneously.

Densitometric Chromatogram Evaluation: Densitometric chromatogram evaluation is done in the reflectance mode with a light beam in the form of a slit

selectable in length and width, which is moved over the sample zones to be evaluated. In order to avoid systematic errors, scanning should always be in or against the direction of chromatography, never at right angles to it. Diffusely reflected light is measured by the photosensor and the difference between the optical signal from the sample free background and that from a sample zone is correlated with the amount of the respective fraction of calibration standards chromatographed on the same plate. Detection (determination) limits for scanning by fluorescence are typically 100 to 1000 times lower than for scanning by absorbance. Therefore, one will always choose the fluorescence mode when substances with inherent fluorescence are to be measured. It is worthwhile to include a pre or post chromatographic derivatization step to render substances fluorescent, when either specificity and/or desired determination limits calls for it. An example is derivatization of primary amines with fluorescamine or other fluorescing agents.

Instead of scanning a chromatogram track with a fixed slit, it is possible to move a light spot in meander form over the sample zones, with a swing corresponding to the length of the slit. This type scanning should offer certain advantages which, however, are sometimes more than paid for with disadvantages. One is the significantly lower signal to noise ratio, the other is lower spatial resolution when the object is an HPTLC plate. In any case, exploiting the advantages of scanning with a moving light spot requires a substantial amount of memory in the data processing unit.

Thin-layer chromatograms are not suitable to be scanned by transmitted light. Apart from undesirable scattering effects, the most serious limitation is the UV cut-off, not only of the supporting glass plate, but of the silica gel itself. The spectral range most suitable for QTLC is UV under 300 nm, as many substances absorb short wave UV without a necessity for derivatization.

Figure 8. Computer controlled TLC evaluation system which consists of Camag TLC scanner II and Hewlett-Packard Model 310 desktop computer printer.

A densitometer for QTLC should be capable of linear scanning by absorbance and fluorescence with a spectral range from 200 to 800 nm. This range can be extended to 190 nm by flushing the monochromator with nitrogen. Raw data processing by a separate peripheral unit allows the user to choose the degree of sophistication, comfort and automation desired. Such a densitometer is shown in Figure (8).

A computing integrator has been considered standard peripheral for some time. But using a personal computer with software tailored for TLC evaluation is becoming more common. The following is a small selection of features that appear suitable to explain the functions of such fully automatic computer controlled system.

a) Automatic peak location and optimization (19)

The scanner searches first in the Y-direction (direction of chromatography) until a signal maximum is detected, then, at this Y-position in the X-direction until that maximum is found. Only then quantification measurement of that fraction is performed.

The user can choose between 4 optimization modes: (1) scanning according to a preselected pattern, without optimization; (2) optimizing the first fraction of the first track, then scanning according to a preselected pattern; (3) optimizing the first fraction of each track, then scanning of the track is rectilinear; and finally (4) optimizing each fraction of the chromatogram.

b) Spectra recording (20)

Absorption as well as fluorescence excitation spectra can be measured. The stored spectral data are used postrun for various purposes selected by the analyst: (1) spectra of individual fractions; (2) superimposed spectra of all fractions in one track, e.g. for identification; (3) superimposed spectra of all equidistantly migrated fractions, e.g. for identity check; and (4) superimposed spectra of different positions within a spot, for checking homogeneity, etc.

c) Multiwavelength scan (21)

Multiwavelength scanning is a way to find the universally useful scanning wavelength for all substances to be quantified, if such exists, Figure (9). Multiwavelength scanning also permits optical resolution of fractions insufficiently separated by chromatography. Another application of multiwavelength scanning is identification of fractions by comparison with standards or against multiwavelengths correlation figures stored in the computer memory. For such purposes it is useful to have the option to measure and compare with up to 9 wavelengths, whereby absorbance and fluorescence may be combined.

Presentation of multiwavelength or multitrack data can be selected, superimposed or three-dimensionally

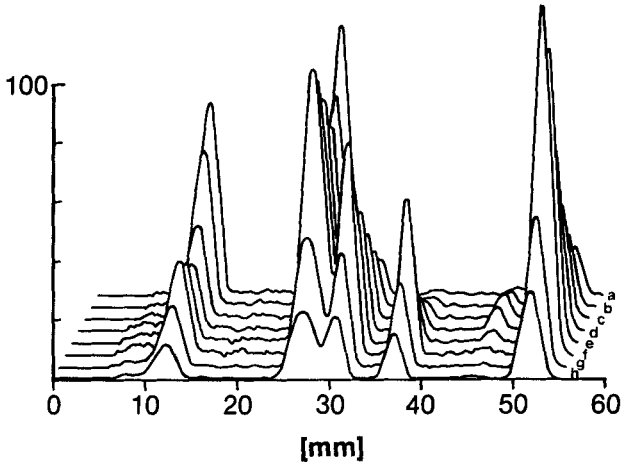

Figure 9. Three dimensional plot of a multiwavelength scans of barbiturates absorbance from 300 nm (h) to 270 nm (a) at 10 nm intervals.

d) Structure subtraction (22)

Another aspect of chromatograph evaluation is structure subtraction. For positive identification and quantification of trace amounts of substances as well as for improving reproducibility, structure subtraction can be employed. The track on the prewashed plate on which the sample shall be chromatographed is scanned and the signal pattern stored. After chromatography, a new scan is taken and structure pattern is subtracted.

Automatic baseline correction, video screen integration, Gauss approximation, evaluation via internal or external standard, one-standard calibration, multilevel calibration, linear or polynominal regression are some other features of the TLC evaluation software.

COUPLING OF HPLC AND TLC

Combining different separation techniques governed by different mechanisms to multidimensional methods is suitable to increase the potential

of the individual techniques (23-25). HPLC is one of the most powerful separation techniques available today for non-volatile substances.

HPLC is most often carried out in the reversed phase mode. Although a considerable choice of reversed phase separation layers is available, as stated earlier and in ref. 26, TLC is most often carried out in the normal phase. It is for these aspects that a combination of HPLC and TLC appears attractive, as it is capable of combining two different separation mechanisms. In addition, the combination of HPLC and TLC gives access to those features that are unique to each technique.

An application example from environmental analysis (27, 28) appears suitable to demonstrate the potential of this coupling technique.

Pesticide residue analyses, e.g., pesticides in water, require identification and quantification of a large variety of substances. More than 50 different components, often in the presence of complex extraneous material. Simultaneous analysis of all these substances side by side would, of course, be preferable over individual analyses for each compound or group of compounds. It is a prerequisite for such analysis, however, that these more than 50 compounds be separated so that they can be reliably identified.

This is not an easy task by either HPLC or TLC. However, it may be possible by a combination of both, i.e. by their direct coupling. This can be achieved by separating first by RP HPLC then spraying fractions of the HPLC effluent directly onto an HPTLC plate which is then AMD chromatographed.

A sample containing 56 pesticide compounds was separated by HPLC on a 2.1 mm microbore column RP-18, 3 µm; gradient elution from water - methanol 95:5 to 5:95, followed by isocratic elution with this solvent. The flow rate was 30 µL/min.

The fraction cuts as shown in the HPLC analog curve, Figure (10) were sprayed onto HPTLC silica gel plates, which were developed by the AMD method. The multiwavelength densitogram of the separation of cut No. 13 is shown as an example, Figure (11).

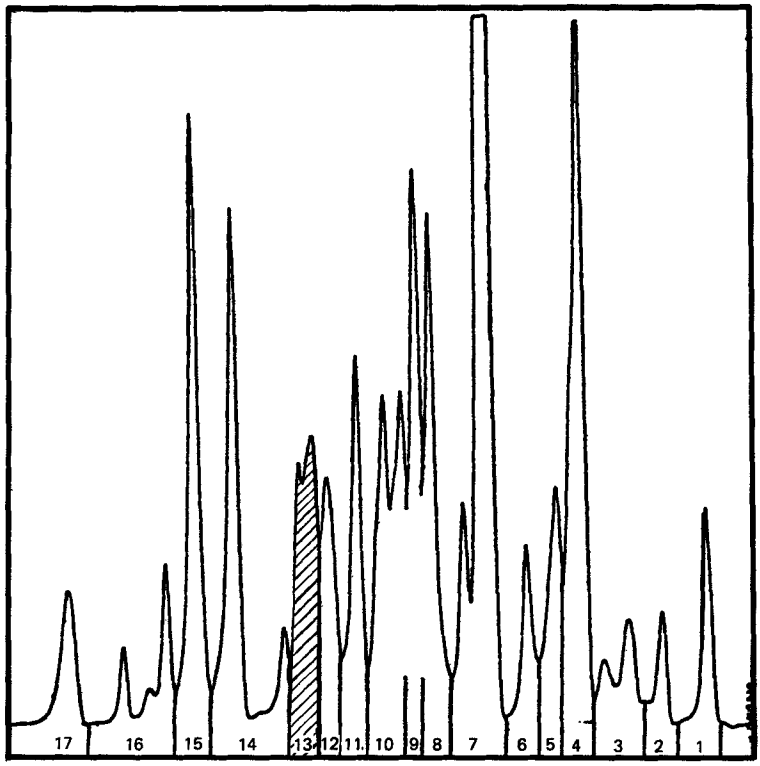

Figure 10. Analog curve of HPLC chromatogram of 56 pesticides, RP gradient elution; hard-cut into 17 fractions as marked.

Systematic scanning of the AMD tracks of all 17 cuts and identifying the fractions by their multiwavelength response revealed all 56 substances. The fact that the combined methods are based on entirely different separation mechanisms is revealed by the fact that in most of these 17 narrow cuts of the HPLC effluent, fractions spread over a wide range of the AMD chromatogram.

This shows that a new dimension in chromatographic resolution can be achieved by coupling reversed phase gradient elution HPLC with normal phase gradient elution AMD-TLC.

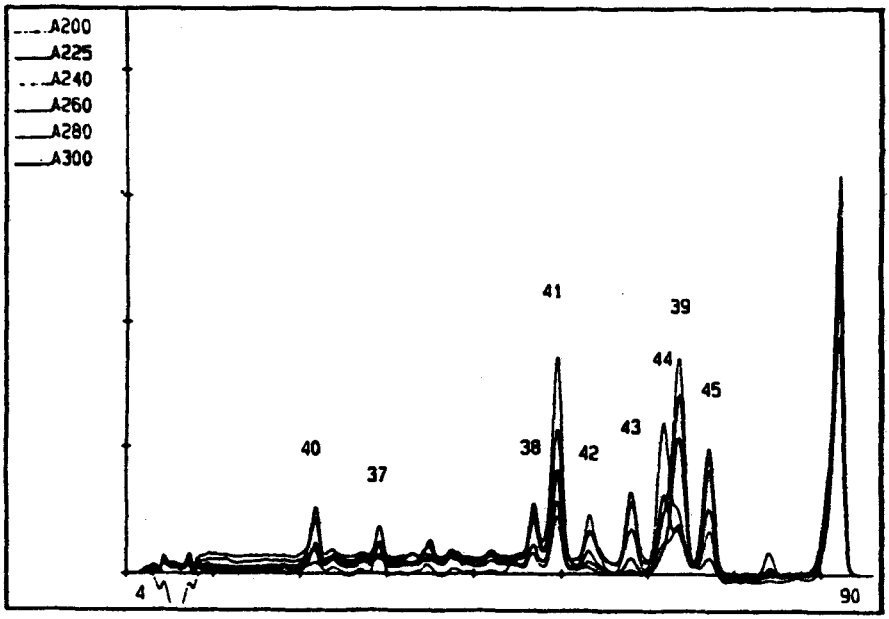

Figure 11. AMD chromatogram of HPLC cut No. 13; a fraction number printed in the plot over a peak indicates that this fraction has been accepted by the software as identified on the basis of multiwavelength response correlation with the respective identification standard.

CONCLUSION

The above discussion shows that HPTLC is a modern, economical, simple, and timesaving analytical technique. HPTLC is competitive and versatile because of its multidimensional properties, the recent introduction of new stationary phases (chiral, cyano-, amino-, reversed phase and side-by-side two-phase), and the wealth of new instrumentation for accurate spotting, developing, spectral measurements and quantification. It is expected that the coupling of both RP HPLC and gradient normal phase TLC will lead to wider applications where complex mixtures need to be resolved.

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