Trends in Thin-Layer Chromatography: 1997

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Abstract

This review deals with the critical evaluation of the present state of the art in the various fields of thin-layer chromatography, the enumeration of possible advantages and disadvantages of the newest developments, prerequisites for the successful application of the newest results, and the prediction of future trends.

Introduction

Thin-layer chromatography (TLC) is a relatively old technique among the various chromatographic separation methods, but it shows some marked advantages over the other chromatographic techniques. In its traditional form, TLC is easy to carry out, does not require complicated instrumentation, and allows the parallel analysis of a considerable number of samples. The selection of the eluents is not limited by the ultraviolet (UV) absorbance, as is the case with many high-performance liquid chromatographic (HPLC) systems, and a wide variety of selective and sensitive chemical and biological detection procedures can be employed. Due to its obvious advantages, the application of various TLC methods for the separation and quantitative determination of a wide variety of organic and inorganic solutes has markedly increased (1,2). Some excellent books (3) and reviews (4) have been published dealing with the various aspects of TLC analysis. Literature on the application and development of new TLC methods is abundant, varied, and fairly scattered. The authors tried to concentrate on the most important studies in the field (a fair number of references can be found in the papers cited) and on the newest techniques recently developed including each step of TLC analysis from sample preparation to

qualitative and quantitative determination of specific solutes.

The objectives of this review are the critical evaluation of the present state of the art in TLC, the enumeration of the possible advantages and disadvantages of the newest developments, the prediction of future trends (necessarily vague), and the more or less impossible daydreams of any scientists dealing with TLC (5,6).

Trends in TLC

Sample preparation

TLC is an adequate technique for the separation and isolation of compounds that are present in a low amounts in complicated organic or inorganic matrices. Polymer matrix components such as carbohydrates, lipid substances, and proteins can deteriorate the efficiency of TLC separation either by moving together with the compounds to be determined or by modifying the retention behavior of the support layer by adsorbing on its surface. Fortunately, TLC plates are disposable; therefore, the sample preparation step is less demanding than in HPLC. Both the traditional liquid–liquid extraction (LLE) (7,8) and the up-to-date liquid–solid (solid-phase) extraction (SPE) (9,10) have been frequently used for the prepurification of samples in TLC analysis. The efficiency of LLE is generally lower than that of SPE. LLE is sometimes time-consuming (the extract generally has to be concentrated in a second step); however, its popularity may be due to the fact that it does not require any additional equipment as SPE does. The selection of solvent or solvent mixtures for both LLE and SPE highly depends on the chemical character of the solutes to be separated and on that of the accompanying matrix. TLC can be used as a pilot method for the selection of optimal SPE conditions; however, it has never been used in this context to our knowledge. Supercritical fluid extraction (11), ultrasonification (12), and microwave extraction (13) considerably increase the efficacy of the LLE step. Surprisingly, these efficient extraction techniques have not been extensively utilized for sample preparation in TLC. Due to their obvious advantages, more frequent application of these approaches can be expected in the future. The wide variety of polar SPE sorbents (silica, Florisil, anion, and cation exchangers), moderately polar SPE sorbents (cyanopropyl, aminopropyl, diol), and apolar SPE sorbents (modified silicas with covalently bonded hydrocarbons on the surface) makes possible the prepurification of any solutes from practically any complex matrices. Although the theory of SPE system selection (sorbent and solvents) for a given prepurification procedure is not entirely clear (14), the practical application of various SPE methods in TLC will grow considerably in the future.

Sample application

Sample application is a crucial step, particularly in quantitative TLC analysis. Due to its paramount importance, much research has been devoted to the development of adequate application devices. The newest results in this field have been recently reviewed (15). The commercial application devices are precise, reliable, and easy to use. They probably will not be replaced in the near future.

TLC sorbents

The appearance (and the subsequent disappearance) of new supports in HPLC is surprisingly rapid (16,17). In contrast, the overwhelming majority of the newest TLC methods use traditional inorganic supports (mainly silica followed with alumina, magnesium silicate, magnesium oxide, diatomaceous earth, etc.), organic supports (cellulose, cellulose derivatives, and polyamide), and organic-coated inorganic supports (octyl-octadecyl-, diol-, and amino-coated silica), which have been well-established for many years in the practice of TLC. However, two new trends can be observed in the use of TLC sorbents. The first and more convenient is the mixing of two or more traditional supports to achieve different retention strengths (which is easy) and/or to achieve different retention selectivity (which is not so easy because the selectivity of one component is generally predominant). Thus, it has been recently reported that the mixture of silica and magnesia has somewhat different retention characteristics than silica, alumina, and Florisil (18,19), and the mixtures of silica–alumina and alumina–cellulose can be successfully used for the separation of rare-earth benzoates (20). The second and, from a chromatographic point of view, more interesting trend is the use of entirely new supports. The number of studies dealing with the testing of new supports in TLC is surprisingly low. It has been recently reported that cerium(III) silicate, a new ion-exchanger, effectively separates metal ions (21). The successful use in HPLC of titanium dioxide (22), zirconia (23), reversed-phase titania (24), and reversed-phase zirconia (25) has recently been reported. Because their retention characteristics are different from those of traditional TLC sorbents such as silica and octadecyl-coated silica, it can be expected that these sorbents will also find application in TLC. Chiral sorbents find only limited application in TLC. This may be due to the fact that each chiral sorbent separates only a limited class of chiral solutes. However, chiral separations have been successfully carried out by the addition of chiral selectors such as cyclodextrins (26,27), cyclodextrin derivatives (28), or other chiral selectors to the eluent (29). The majority of studies used amino acids or amino acid derivatives for the evaluation of the separation capacity of various chiral selectors. The influence of β-cyclodextrin (β-CD) on the separation of dansyl-amino acid enantiomers on octadecylsilane plates is demonstrated in Table I. It was established that the method separates both mono- and di-dansyl derivatives of 19 common amino acid enantiomers except proline and tryptophan (26). α-CD is also suitable for the separation of enantiomeric amino acids, as demonstrated in Table II. It was emphasized that the separation efficiency of the enantiomer depends greatly on the composition of the mobile phase and on the chemical structure of the solutes. The growing acceptance and application of these types of chiral separations can be expected in the future. As cyclodextrins and cyclodextrin derivatives form different inclusion complexes with different enantiomers of a large variety of compounds, their use as a mobile phase additive for chiral separation in TLC is highly recommended. TLC analysis is generally limited for the separation and quantitative determination of solutes with low molecular mass. However, there would be considerable interest in the development of sorbents (or simply use the sorbents applied in HPLC for the same

Table I. Reversed-Phase TLC Data for the Separation of Dns-Amino Acids

Solvents: A = MeOH–0.20M β-CD (35:65, v/v); B = CH_3CN–0.20M β-CD (32:68, v/v); C = CH_3CN–0.20M β-CD (20:80, v/v); D = MeOH–0.20M β-CD (55:45, v/v); E = MeOH–saturated β-CD (60:40, v/v); F = MeOH–0.20M β-CD (50:50, v/v).

Dns-Amino acid	Abbreviation	$R_{F(D)}$	$R_{F(L)}$	α*	R_s†	Solvent‡
DL-Alanine	Dns-DL-Ala	0.47	0.40	1.43	1.64	A
DL-allo-Isoleucine	Dns-DL-allo-Ile	0.38	0.30	1.43	3.25	B
DL-Asparagine	Dns-DL-Asn§	0.69	0.60	1.39	1.53	C
DL-Arginine	Dns-DL-Arginine	0.65	0.55	1.52	1.69	C
DL-Citrulline	Dns-DL-Cit	0.63	0.54	1.45	1.52	C
DL-Cystine	*N,N'*-Di-Dns-DL-Cys-Cys	0.42	0.37	1.23	1.52	D
DL-Glutamine	Dns-DL-Gln	0.66	0.57	1.46	1.86	C
DL-Histidine	*N*-(α)-Mono-Dns-DL-His	0.64	0.58	1.28	1.13	C
	N-(α),N-(im)-Di-Dns-DL-His**	0.22	0.19	1.20	0.94	E
DL-Isoleucine	Dns-DL-Ile	0.40	0.33	1.35	1.71	B
DL-Lysine	*N,N'*-Di-Dns-DL-Lys	0.39	0.35	1.19	1.02	E
N-Methyl-DL-valine	Dns-DL-*N*-Me-Val**	0.28	0.24	1.18	0.94	F
DL-Ornithine	*N,N'*-Di-Dns-DL-Orn	0.40	0.35	1.24	1.20	E
DL-Pipecolic acid	Dns-DL-Pip	0.25	0.25	1.00	0	F
DL-Proline	Dns-DL-Pro	0.41††	0.39††	1.10††	0.63††	F
DL-Tyrosine	*N,O*-Di-Dns-DL-Tyr	0.26	0.23	1.15	0.94	E

* $\alpha = [1 - R_{F(L)}/R_{F(L)}] / [1 - R_{F(D)}/R_{F(D)}]$.
† R_s = 2 (distance between the two spots) / (sum of the widths of the two spots).
‡ Solutions contained urea and sodium chloride.
§ Part of the D-spot began to elute near the solvent front. The main body of this spot was used for these calculations.
** The identity of the D- versus L-spot was not confirmed for this derivative.
†† These are only approximate values because of overlap of the spots.

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purposes) for the TLC separation of bioactive macromolecules such as peptides, proteins, and perhaps carbohydrates.

Development and development optimization

Many different techniques were used for the development of TLC plates. The advantages and disadvantages of linear ascending development, linear horizontal development, circular development, and anticircular development and the corresponding semiautomated and automated developing instruments suitable for carrying out such developments have been recently reviewed (30). As the efficacy of separation processes depends considerably on the appropriate choice of stationary and mobile phase, the optimization of the separation process has a marked impact on the efficacy of any TLC analysis. Thus, a computer-assisted method was developed for the selection of the mobile phase on the basis of the Snyder theory (31), and the importance of the exact determination of optimization criteria has recently been discussed (32). Numerous examples demonstrated that two-step or multistep development results in a considerable increase in separation efficiency (33,34). The successful use of multistep development is demonstrated in Figure 1, which represents the separation of the extract of *Radix rhei* on silica support (34). Different computer-aided optimization methods were developed for both two-step (35) and multiple developments (36) in

Table II. Enantiomeric Separation of Aromatic Amino Acids and Aromatic Amino Alcohols

Compound	R_{F1}	R_{F2}	α	R_s
DL-Tyrosine	0.87 (D)	0.71 (L)	1.21	3.76
	0.89 (D)*	0.79*	1.13*	1.72*
DL-3,4-Dihydroxyphenylalanine (DOPA)	0.83 (D)	0.72 (L)	1.15	3.76
DL-*p*-Hydroxyphenylglycine	0.87	0.62	1.40	
DL-Thyronine	0.88	0.77	1.14	
DL-*p*-Aminophenylalanine	0.82	0.73	1.12	
DL-Epinephrine	0.76 (L)	0.76 (D)	1.12	
DL-Isopropylepinephrine	0.97	0.97	1.00	
DL-Phenylalanine	0.80	0.80	1.00	
DL-Tryptophan	0.74	0.74	1.00	

Mobile phase: methanol–formic acid–0.2M α-CD solution of urea (7:1:2). Stationary phase: cellulose TLC plates. Temperature: 25°C, pH 4.5.

*Mobile phase: methanol–0.2M α-CD solution of urea–33% diethylamine (4:1:0.42).

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TLC. Gradient methods in TLC are more or less successful imitations of the real gradient elution in HPLC. Unlike in HPLC, the gradient development in TLC is not continuous and is always linear. The development and commercialization of an apparatus suitable for the continuous (rather than linear) change of eluent composition would be a considerable step toward increasing the efficacy of TLC. The general weakness of the optimization methods is the arbitrary selection of the preliminary conditions (i.e., selection of silica as the stationary phase when the same separation could be more efficiently carried out on alumina or octadecylsilica supports, selection of two or three solvents when other solvents may show better separation characteristics, etc.). After the preliminary conditions are fixed, the optimization methods will certainly find the optimum. However, it is a local optimum, and the chromatographer can never be sure that it is near the theoretical absolute optimum. The development of optimization methods including the most efficient preselection of support and the solvent components is not possible with the present state of knowledge. If such a scheme could be developed, however, it would greatly facilitate the rational design of TLC separations.

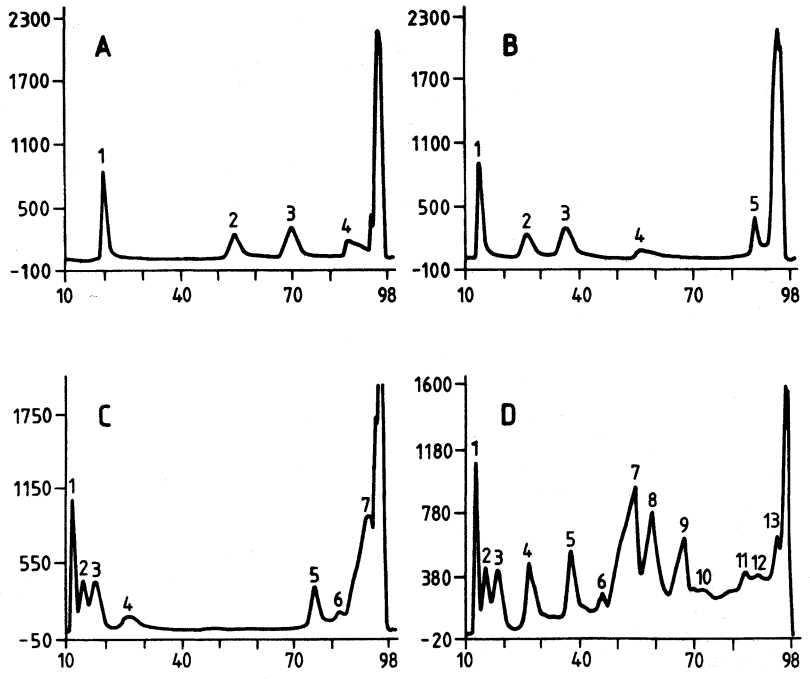

Figure 1. Densitograms of the extract from *Radix rhei*. (A) First development, 10% (v/v) EtOAc + $CHCl_3$, distance: 9 cm. (B) Second development, 50% (v/v) EtOAc + $CHCl_3$, distance: 9 cm. (C) Third development, 100% EtOAc, distance: 8 cm. (D) Fourth development, 15% (v/v) MeOH + EtOAc, distance: 5 cm. *(Reprinted with permission from reference 34.)*

Solute detection and identification

Detection in TLC is generally based on the UV or visible absorption of the solutes or on the use of various detection reagents. One of the main advantages of TLC is that a high number of detection reagents can be used that are more or less selective. The use of nonselective reagents has some advantages; each spot on the plates can be detected and identified at the next detection step by using more selective reagents. The development of

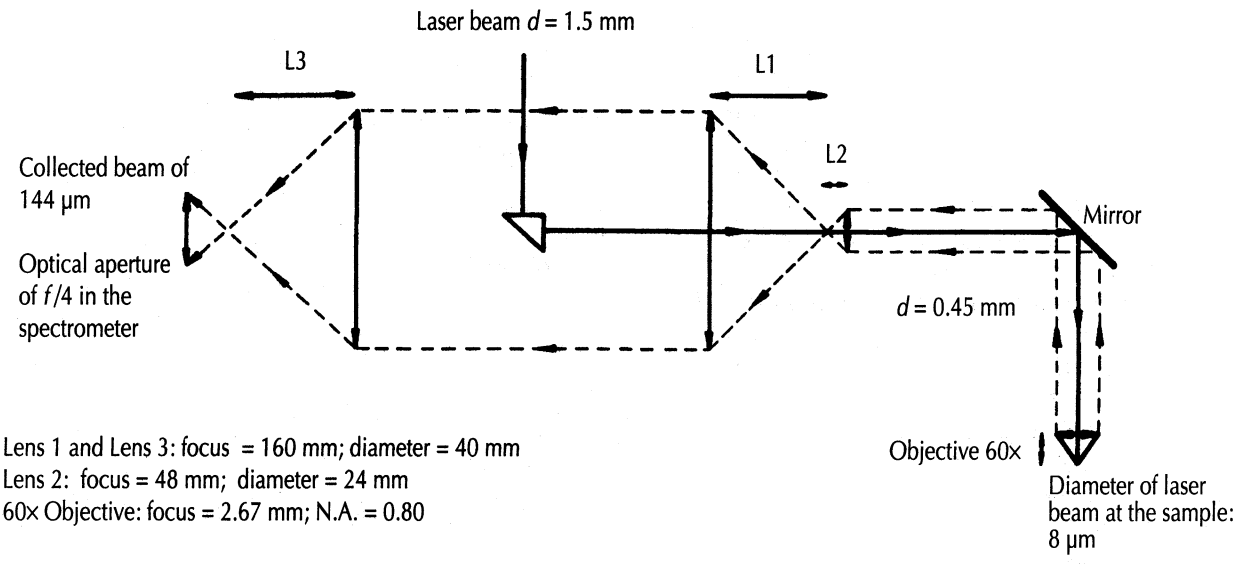

Figure 2. Optical layout of the interfacing elements of an FT Raman microspectrometer with a two-lens collection system. The objective focuses the laser beam onto the sample and refocuses the scattered light at the entrance of the spectrometer via the three lenses. *(Reprinted with permission from reference 37.)*

new reagents for TLC is continuous. The requirements for future reagents are either higher selectivity or the simplicity of the detection reaction (lower reaction temperature, less corrosive and toxic components, etc.).

Similar to the other modes of chromatography, the identification of solutes is controversial in TLC as well. It is tacitly assumed that compounds having the same retention are identical. Theoretically this assumption is untenable. Having the same retention makes it probable that compounds are identical, but such an assumption does not prove the identity or equality of solutes. Moreover, pure standards are needed to identify unknown compounds. Methods other than TLC are required for the identification of compounds. Theoretically, solute-specific reagents are suitable for such identification. However, the number of reagents that are specific for only one compound is negligible. The use of most detection reagents helps in the identification of the type or class of compounds but not the individual compounds themselves. The unique solution to identification is the use of combined methods. TLC has been combined with many other methods such as FT Raman and FT SERS (surface-enhanced Raman scattering) microscopy (37), matrix-assisted laser desorption/ionization mass spectrometry (MALDI) (38), Fourier transform infrared (FTIR) (39), square-wave stripping voltammetry (SWASV) (40), and tandem mass spectrometry (MS–MS) (41). The setup of the FT Raman and FT SERS microspectrometry is shown in Figure 2. It has been found that this experimental setup allows the identification of solutes at the sub-femtogram level and the use of plates with fluorescence indicators. The scheme of the TLC–MALDI procedure is illustrated in Figure 3. The sensitivity of this method is fairly high, as demonstrated in Table III. The spatial resolution of the method was between 250 and 500 µm, and the absolute detection limit was in the picogram range, which indicates that this method can be used for the accurate imaging and evaluation of TLC plates. The schematic diagram of the on-plate TLC–SWASV is shown in Figure 4. This method has proven to be successful in the detection of Cd(II), Cu(II), and Pb(II) at nanogram levels after TLC separation. The validation parameters of the method were acceptable, as demonstrated in Table IV. The use of MS–MS using two mass spectrometers connected in series allows more precise identification of solutes. The block diagram of an MS–MS arrangement is shown in Figure 5. The methods coupling TLC with spectroscopic techniques allow not only the more precise determination of the spot position but also the accurate identification and quantitation of the solute (42). The detection limits of various solutes determined by combined methods are compiled in Tables V and VI. Combined methods represent the future of modern TLC. They are more sophisticated, and they increase enormously the information content obtained from one TLC separation.

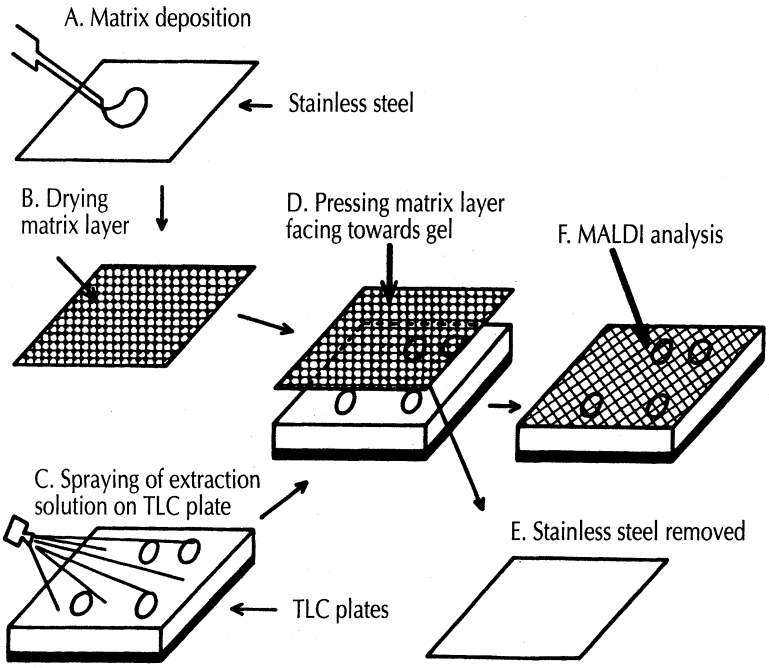

Figure 3. TLC–MALDI coupling procedure. *(Reprinted with permission from reference 38.)*

Table III. Matrix-to-Analyte Ratios Obtained for Different Quantities of Angiotensin II and Methods of Matrix Deposition

	Quantity of analyte	
Method of analysis	**2 ng**	**Detection limit**
Indirect matrix deposition on TLC plate	$2 \times 10^{5*}$	$2 \times 10^{6*}$ (0.2 ng)
Direct matrix deposition on TLC plate	$4 \times 10^{6*}$	$4 \times 10^{6*}$ (2 ng)
Conventional MALDI analysis	$1 \times 10^{4\dagger}$	$4 \times 10^{6\dagger}$ (5 pg)

* For 5 × 4-mm analyte spot.
† For 2 x 2-mm analyte spot.

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TLC as a pilot method for HPLC

TLC and HPLC are similar in many aspects. Both of them use a stationary and a mobile phase, and the solutes are separated according to differences in their affinities to the phases. It has often been indicated that TLC can be successfully used for the prediction of solute retention behavior in HPLC (43,44). However, the most important prerequisite of the transfer of TLC retention data to HPLC is the similarity of the supports. It is especially relevant in adsorption systems where the different surface pH of the TLC and HPLC supports may result in entirely different retention orders for acidic or basic compounds. Often ammonia or ether (i.e., volatile components) in a mobile phase causes problems when going from TLC to HPLC. In our opinion, the main advantage of using TLC as a pilot method for HPLC is the possibility of detecting compounds that do not migrate or show negligible mobility in the eluent system. These nonmoving or slow-moving solutes can cause considerable

concern to the chromatographer. The nonmoving component can slowly contaminate a column if not removed frequently. The slow-moving components will elute in their own time, usually in the middle of later sample runs, looking like a drifting base line. Using various TLC detection methods, the presence of impurities not detected in HPLC can be also verified.

Determination of molecular parameters

TLC can be used not only for the separation and quantitative determination of a wide variety of compounds but also for the measurement of chemical and physicochemical parameters of different compounds. As the retention in both adsorption and reversed-phase TLC is governed by the physicochemical characteristics of the solutes (hydrophobicity, hydrogen-donor and hydrogen acceptor capacity, sterical dimensions, etc.) and by those of the eluent components (dielectric constant, dipole moment, and other polarity parameters), some physicochemical parameters of solutes and eluent components can be calculated by measuring the retention under strictly controlled conditions. Thus, adsorption TLC has recently been used for the determination of solvent polarity using selected nitro-containing solutes (45). It has been further established that the pK_a values of isomeric methylphenols, chlorophenols, and nitrophenols can be calculated from their topological indices and the R_M value ($R_M = \log [1/R_f - 1]$) (46). Lipophilicity is a molecular parameter extensively used in the computer-assisted design of new bioactive compounds (47). Among other chromatographic methods such as gas–liquid chromatography and reversed-phase HPLC, reversed-phase TLC has also been frequently employed for determining the hydrophobicity of various bioactive compounds such as xanthine and adenosine derivatives (48) and nonhomologous series of antibiotics (49). Reversed-phase TLC can also be used for determining the relative strength of interaction between various molecules. The latest new developments in this field were recently reviewed (50). The advantages of TLC for measuring physicochemical parameters are the possibility of the parallel determination of one or more characteristics of a fairly large number of solutes, the simplicity of the experimental setup, and the speed of the measurement. The disadvantages are the relatively high standard deviation inherent in each TLC method and the necessity for the calibration of the method with other well-established separate chemical and physicochemical procedures. However, one must keep in mind that relative standard deviations of other nonchromatographic methods used for the determination of various molecular parameters are sometimes equal or higher than that of TLC techniques. The number of papers dealing with the application of various TLC techniques for the determination of molecular parameters is continually increasing, and the wide-spread use of these simple, rapid, elegant methods can be expected in the future.

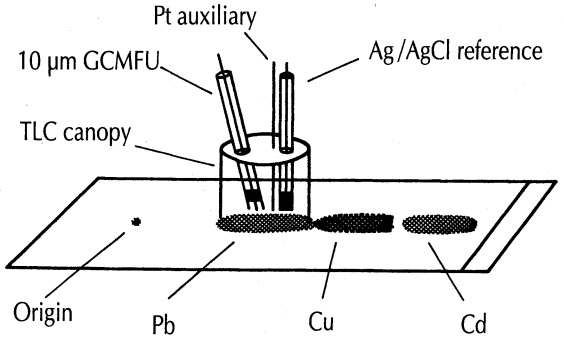

Figure 4. Schematic diagram of the on-plate TLC–SWASV apparatus. *(Reprinted with permission from reference 40.)*

Table IV. Summary of Within-Spot (Three Replicates) and Spot-to-Spot Reproducibility for 100-ng Pb Samples Quantitated by TLC–SWASV

Spot no.	Peak height (nA)	Standard deviation (nA)	RSD (%)
1	4.30	0.22	5.16
2	6.13	0.12	1.99
3	5.94	0.22	3.65
4	3.62	0.03	0.85
Total	5.00	1.12	22.5

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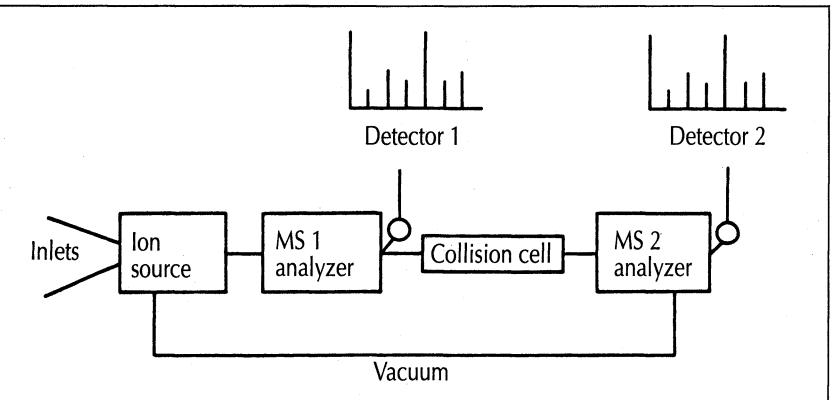

Figure 5. Block diagram showing the typical arrangement of a tandem spectrometer. *(Reprinted with permission from reference 41.)*

Quantitation and validation in TLC

TLC has been developed as a qualitative method for the separation of various organic and inorganic compounds from complicated matrices and for the detection of impurities in synthetic products. TLC in its original form is suitable only for semiquantitative analysis, visually comparing the spot of solute with the spots containing well-defined amounts of the solute. With the development and commercialization of various TLC scanners and softwares, accurate quantitative analysis has also become a reality in TLC. The various aspects of the quantitative analysis in TLC have recently been reviewed (51). The newest versions of TLC scanners show higher sensitivity than the older ones; however, a significant breakthrough (appearance of a

Table V. Compounds Analyzed by in situ TLC–FTIR*

Compound	Sorbent	FTIR mode	Detection limit (µg)
Amaryllidaceae alkaloids	Sil	drift	0.15
Amino acids	Sil, Alu	transmission	0.5†
Analgesics	Cel	drift	0.2
	Sil	drift	0.1
	Sil	drift	0.1†
Benzodiazepines	Sil	drift	0.5†
Diazonaphtoquinones	RP	drift	0.4
Dyes	Sil, Alu	transmission	0.2†
	Alu	transmission	0.01
	Sil	drift	1†
	Zir	drift	0.3
	Zir	drift	0.01
EDTA	Sil	drift	0.25
PAHs	Zir	drift	1
Pesticides	Alu	transmission	10
	Alu	transmission	0.01
Pharmaceuticals	Sil, Alu, Cel, RP	drift	1†
Phenylureas	Sil, Alu, Cel, RP	drift	50†
Phtalates	Sil, RP	drift	1
Surfactants	Sil	drift	50†

* Abbreviations: Sil = silica; Alu = alumina; Cel = cellulose; RP = reversed-phase; Zir = zirconia.
† Estimated on the basis of reported data.
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Table VI. Compounds Analyzed by Transfer TLC–FTIR*

Compound	Transfer method†	FTIR mode	Detection limit (µg)
Amino acids	Shimadzu	drift	2‡
Benzodiazepines	scrape + wick–stick	transmission	20‡
Coricosteroids	Chromalect	drift	2‡
Dyes	scrape + extr.	drift	0.01‡
	in situ extr. + wick–stick	transmission	1‡
	Chromalect	drift	1‡
Esters and ketones	TGA–FTIR	transmission (vapor)	1
PAHs	Eluchrom	transmission	1‡
Pharmaceuticals	Shimadzu	drift	0.2‡
Phenols	scrape + extr.	transmission	2‡
Phtalates	scrape + extr.	drift	0.05‡
Phospholipids	Shimadzu	drift	0.1‡
Polymer additives	scrape + wick–stick	drift	1‡
	in situ extr.	drift	10
Water constituents§	scrape + extr.	transmission	5

* PAHs = polycyclic aromatic hydrocarbons.
† Transfer methods: Shimadzu = using TLC–FTIR accessory of Shimadzu; Chromalect = TLC–FTIR accessory of Analect; scrape + extr. = spot scrape off followed by solvent extraction and evaporation on KBr; scrape + wick–stick = spot scrape off followed by wick–stick technique; in situ extr. + wick–stick = in situ spot extraction followed by wick–stick technique; TGA–FTIR = thermogravimetric FTIR analyzer; Eluchrom = Eluchrom system (Camag, Muttenz, Switzerland); in situ extr. = in situ spot extraction followed by evaporation on KCl.
‡ Estimated on the basis of reported data.
§ Constituents of surface water, waste water, sludge, and sediments.

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revolutionary new scanner) cannot be expected in this field in the near future.

Validation is a prerequisite of any reliable chromatographic analysis (52). Many chromatographic parameters have been proposed for inclusion in the validation process, such as linearity of the calibration curve (53), sensitivity and selectivity of solute detection, interday and intraday reproducibility (54), instrument precision (55), detection limit (56), quantitation limit (57), recovery (58), ruggedness (59), etc.

Unfortunately, validation processes are not frequently used in quantitative TLC analysis, which makes the reliability of the results questionable. The growing demand for the validation of analytical methods (pharmaceutical compendial and regulatory methods require validation) will force the use of validation processes in TLC in the future.

Acknowledgment

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