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Planar Chromatography

Joseph Sherma

Department of Chemistry, Lafayette College, Easton, Pennsylvania 18042

Review Contents

This review covers the literature of thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) found by computer-assisted searching in *Chemical Abstracts* and the *ISI Web of Science* from November 1, 2005 to November 1, 2007. The literature search was augmented by consulting *Analytical Abstracts*, and the following journals that regularly publish papers on TLC were searched directly: *Journal of Chromatography* (parts A and B and the bibliography issues), *Journal of Chromatographic Science*, *Chromatographia*, *Analytical Chemistry* , *Journal of Liquid Chromatography & Related Technologies*, *Journal of AOAC International*, *Journal of Planar Chromatography-Modern TLC*, *Acta Chromatographica*, and *Acta Universitatis Cibiniensis, Seria F, Chemia . Chemical Abstracts* (SciFinder Scholar) cited 2290 references containing the search phrase "thin-layer chromatography" during the review period, compared to 1978 citations for the last review period, November 1, 2003 to November 1, 2005; the actual totals are higher than these numbers because all worldwide journals publishing papers on TLC are not surveyed by this service. No papers reporting new research on paper chromatography, the other main classification of planar chromatography, were considered to be important enough to be included in this review.

Coverage is limited to the most significant papers representative of the current practice and significant advances in the field of TLC, with specific sections on history and literature, fundamental studies, methodology, equipment, and instrumentation. TLC continues to feature a broad range of applications, such as analysis of pharmaceuticals, herbal medicines and dietary supplements, biological and clinical samples, foods and beverages, environmental pollutants, and chemicals, but because of a prescribed limitation of approximately 250 references for this article, there are no sections covering individual compound classes, as in my biennial planar chromatography reviews published continually in this journal from 1970 to 2002. However, important new applications to specific analytes and sample matrices of many types are cited in all sections throughout this review, especially in situ densitometric methods in the Quantitative Analysis section.

The presentations at the International Symposium on Thin Layer Chromatography, held in Berlin, Germany, in October, 2006, and the papers contained in two special journal issues provide an up-to-date picture of the some of the most important technique and application areas in TLC research over the past 2 years. The symposium began with a workshop by Dr. Gerda Morlock that described the methodological steps of HPTLC, including pretreatment of the plate and sample application, mobile phase optimization, controlling the development chamber environment, visualizing substances on a plate (benefits of multiple detection), chemical and biological detection, densitomteric techniques, and confirmation of results by mass spectrometry (MS). Symposia were held on fundamentals and theoretical aspects, food analysis, plant analysis, clinical applications, bioactivity testing, and coupling methods, and there were 53 poster presentations on instruments, methods, and applications. The 2008 International TLC Symposium will be in Helsinki, Finland on June 11−13.

Subjects of papers in a special issue on TLC of the *Journal of Liquid Chromatography & Related Technologies* [**2006**, *29* (14)], guest edited by Sherma and Fried, included retention behavior of some thiophosphorylglycinamide fungicides by adsorption and reversed phase (RP) TLC; use of RP-HPTLC for determination of the lipophilicity of 3,5-dioxo-4-azatricyclo[5.2.2.02,6]undecanes-5-HT_{1A} antagonists; influence of the temperature of silica gel activation on separation of selected biologically active steroid compounds; automatic selection with LSChrom software of mobile phases for the silica gel TLC of libraries of piperidones; TLC investigation of oscillatory instability of selected Profen enantiomers in physiological salt; mechanism of oscillatory changes of the retardation factor value (R_f = distance of zone migration/distance of mobile phase migration) and specific rotation with selected solutions of *S*-(+)-naproxen; TLC-direct bioautography of flumequine antibiotic residues in milk; preparative rotation planar chromatography for isolation of ecdysteroids; qualitative and quantitative analysis of phospholipids by Iatroscan (Mark-6s) TLC with flame ionization detection (TLC-FID); simultaneous determination of fusidic acid, *m*- and *p*-hydroxybenzoates, and butlyhydroxyanisole by TLC−ultraviolet (UV) densitometry; HPTLC identification of green tea and green tea extract; TLC analysis of corrinoid compounds in a lactic acid bacterium; and studies by HPTLC on RP chemically bonded C-18 (also called RP-18) silica

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gel and unmodified silica gel plates of the effects of estivation and starvation on lutein, β-carotene, and neutral and polar lipid concentrations in two species of medically important snails.

Papers in another special issue on TLC of the same journal [**2007**, *30* (15)], also guest edited by Sherma and Fried, had papers on automatic mobile phase selection for silica gel and alumina TLC of dibenzo[*c*,*h*]chromen cis/trans isomers; use of a modified printer for application of detection reagents; studies of the enantioseparation and oscillatory transenantiomerization of *S*,*R*-(+/−)-ketoprofen on silica gel layers with densitometric detection; quantitative silica gel HPTLC analysis of artemisinin in dried *Artemisia annua* L. leaves; studies of TLC systems and detection methods for selected carboxylic acids; separations of cephalosporins on silica gel and cyano, amino, and diol bonded silica gel layers; silica gel TLC-bioautography analysis of a corrinoid compound from dark muscle of yellowfin tuna; separation of selected flavonoids by RP-high performance column liquid chromatography (HPLC) coupled with normal phase (NP)-HPTLC; use of densitometric analytical and preparative TLC to elucidate the lipid composition of raw and microwaved roasted walnuts, hazelnuts, and almonds on layers of plain silica gel and silica gel modified with silver nitrate or dimethyldichlorosilane; comparative study of the separation and quantification of lipid classes by TLC−FID on Chromarods A and SIII; densitometric study of the separation of nicotinic acid and its amides on alumina and RP-18WF plates (W indicates a water wettable layer, F a layer with a phosphor fluorescing at 254 nm); and determination of sterols and fatty acids in prostate health dietary supplements by HPTLC−visible mode densitometry.

A recent article (*1*) discussed the increasing infiltration of counterfeit drugs into markets worldwide and the analytical techniques being used to identify them. TLC was named as the main method for qualitative and quantitative analysis in the field, mostly by use of the Minilab kit, developed by the German Pharma Health fund (GPHF).

A bibliography service (CBS) is offered by Camag Scientific Inc., Wilmington, NC, to keep subscribers informed about publications involving TLC. This service is available from Camag free of charge in paper format, or abstracts can be downloaded from their Web site http://www.camagusa.com and searched by keyword (author name, analyte, sample, technique, reagent, etc.). In addition to a review of the literature and descriptions of new products, each issue of the Camag CBS contains a section on HPTLC applications, e.g., determination of amitrole in water, monitoring of oxytetracycline dose in medicated salmon feed, quantification of isopropylthioxanthone (ITX) in food with coupling to electrospray ionization mass spectrometry (ESI-MS) and direct analysis in real time MS (DART-MS), and determination of glibenclamide adulteration in herbal drugs [issue 96; March, 2006]; structural characterization of gangliosides by matrix assisted laser desorption/ionization (MALDI) MS using an infrared (IR) laser and time-of-flight (TOF) MS mode, assessing the authenticity of absinthe, validated analysis of the biomarker trigonelline, and identification of green tea and green tea extract [issue 97; September, 2006]; and determination of amino-propanol in dermatological products, stability testing of gatifloxacin, and analysis in polymeric nanoparticles, chlorine-free mobile phase for determination of polycyclic aromatic hydrocarbons (PAHs) in water extracts, and evidence of methyl and desphenyl metabolites of chloridazon in water samples [issue 98; March, 2007].

Many additional applications are available on the Camag Web site. Camag also publishes articles titled "CAMAGflash" on its Web site, and subscribers can automatically receive them by e-mail; the three published so far have described HPTLC services offered by the Camag Laboratory, such as feasibility studies and contract analyses [March, 2007 issue]; cGMP (good manufacturing practices) in HPTLC botanical analyses [July, 2007]; and HPTLC as an orthogonal technique complementary to HPLC [October, 2007].

The death of Prof. Szbolics Nyiredy on October 30, 2006 was noted in an obituary (*2*) that outlines his vast scientific accomplishments, most notably in research, writing, and organization of meetings in the field of TLC. Prof. Nyiredy was the editor-in-chief of the *Journal of Planar Chromatography-Modern TLC* from its first issue in February, 1988 until his death. Starting with volume 20, issue no. 1 (February, 2007), the new publisher of this journal is Akademiai Kiado (A.K.), Budapest, Hungary, and the new editor-in-chief is Prof. Bernd Spangenberg, University of Applied Sciences, Offenburg, Germany.

HISTORY, STUDENT EXPERIMENTS, BOOKS, AND REVIEWS

Siouffi gave an account of evolution of TLC starting in the 1940s, with a focus on the plates, techniques, commercial equipment, multidimensional capability, and detection modes. This otherwise excellent article contains a table that lists the number of articles published on planar chromatography in selected years from 1990 to 2004 as being in the range of 210−490, which are certainly below the actual numbers published in that period (*3*).

TLC was used to identify an unknown analgesic product by comparing R_f values of its components with those of known standards in a student experiment suitable for an organic chemistry laboratory course (*4*). A student experiment of medical importance was described involving extraction of lipids from simulated amniotic fluid samples followed by separation, detection, and densitometric scanning of the lecithin and sphingomyelin bands on TLC plates (*5*). A method for students to quantify photosynthetic pigments in green beans was based on TLC with silica gel and cyclohexane−acetone−diethylamine (10:4:1) mobile phase and densitometry using a CanoScan 5200F flatbed scanner and IGOR Pro image analysis software (*6*).

A general book by Wall on TLC procedures that is part of the RSC Chromatography Monographs series contained chapters on history, sorbents and layers, sample pretreatment, sample application, development techniques, detection of zones, quantification and video imaging, and coupling techniques (*7*). The second edition of the practical book by Hahn-Dienstrop is especially applicable to pharmaceutical analysis under good manufacturing practices (GLP/GMP regulations) (*8*). Reich and Schibli wrote a detailed, practical guide to the HPTLC qualitative and quantitative analysis of medicinal plants, which contains a large number of beautiful and very informative colored photographs of plant extract chromatograms (*9*). Kowalska and Sherma edited the first books entirely focused on classical preparative layer chromatography (PLC) (*10*) and TLC in chiral separations and analysis (*11*). The book titled *Thin Layer Chromatography in Phytochemistry*, edited by Waksmundzka-Hajnos, Sherma, and Kowalska, was published

by CRC Press in 2008; this is the first book to cover all aspects of the TLC determination of primary and secondary plant metabolites.

A journal editorial emphasized some of the advantages and complementary aspects of TLC compared to HPLC, such as simplicity and low cost, which allow its successful use in many situations such as by operators in a manufacturing plant; ability to detect everything in the sample with appropriate visualization procedures without the possible losses that can occur because of retention on a column; ability to obtain quantitative data; and preparative isolation of separated compounds (*12*). The experimental steps involved in modern quantitative HPTLC were reviewed with selected examples of applications (*13*).

As mentioned above, a comprehensive review of TLC applications to specific compound classes is not possible in this article because of space restrictions. However, information on TLC/HPTLC applications is available in the following review articles: lipids in snails as affected by diet and larval parasitism (*14*); vitamins and their derivatives in diet supplements and foods (*15*); amino acids and short peptides (*16*); multidimensional separations including TLC for biomedical and pharmaceutical compounds (*17*); testing of the purity of pharmaceuticals (analysis of pharmaceuticals and herbal medicines is the most frequently reported application of TLC) (*18*); biogenic amines in foods (*19*); phospholipids in biological samples (*20*); insecticides, herbicides, and fungicides in food, crops, biological, environmental, pharmaceutical samples and formulations (*21*); mycotoxins (*22*); natural product based supplements, such as ginseng (*23*); and zeranol residues in animal products (*24*). Additional published review articles are cited in the pertinent sections below.

THEORY AND FUNDAMENTAL STUDIES

This section contains a selection of papers reporting theoretical and fundamental TLC studies that were chosen as examples of some active research areas. These references illustrate the different types of layers and mobile phases used for TLC at this time.

The following are examples of the many studies of retention mechanisms and compound separations that were reported: review of quantitative structure−chromatographic retention relationship (QRRS) studies covering the period 1996−August, 2006, with a summary of general tendencies, misleading practices, and conclusions, validation of the models, suggestions for future work, and applications (*25*); QRRS developed for a series of (*Z*)- and (*E*)-2-allcylidene-4-oxothiazolidine derivatives by multiple linear regression (MLR) analysis with measurement of lipophilic character of analytes by RP-TLC (*26*); partial least-squares study of the effects of the mobile phase (aqueous ammonia−organic modifier, acetonitrile (ACN), dioxane, or acetone) and physicochemical properties on the retention of some thiazoles on RP C-18 bonded layers (*27*); prediction of the R_M retention values [$R_M = \log (1/R_f - 1)$] in 13 TLC screening systems on silica gel by a novel atomic contribution system (*28*); retention behavior of uroporphyrins I and III, chloroporphyrins I and III, and protoporphyrin IX on silica gel, polyamide, and cellulose plates with a homologous series of *n*-alcohols, ACN, and tetrahydrofuran (THF) as mobile phase (*29*); effect of nonaqueous mobile phase composition on the retention of macrocyclic antibiotics on a C-18F plate with mixtures of alcohols with dimethyl sulfoxide (DMSO) or hexamethyldisiloxane as mobile phases (*30*); and development of a model for retention in NP-TLC with ternary mobile phases that uses the difference in adsorption of a solute and all solvents onto the solid surface as the driving force and a series of simple generated linear relationships to predict the retention factor (*31*).

A very active research area involves the determination of compound lipophilicity (or hydrophobicity) by RP TLC quantitative structure−activity relationship (QSAR) studies with the goal of determining a compound's biological activity for use in modern structure based drug design. For example, lipophilicity is considered to have a dominant role in drug penetration through hydrophobic cell membranes and uptake by target organs or organisms via drug receptor binding. The chromatographic behavior of polyphenols (flavonoids and phenolic acids) on C-18F layers with binary mixtures of methanol (MeOH) and phosphate buffer as the mobile phase was correlated with the compound's lipophilicity, solubility, plasma−protein binding, and oral absorption (*32*). The lipophilicity of seven bile acids was determined by RP-TLC on chemically bonded cyano (CN) F, C-18W, and C-2 layers with mobile phases composed of water mixed with MeOH, ACN−MeOH, acetone, or dioxane; the most significant correlation was found between R_{MW} and φ(O) lipophilic parameters and partition coefficient (log P_{KOWIN}) values (*33*). The following are additional TLC lipophilicity studies carried out by RP-TLC on chemically bonded C-18 (RP-18) plates unless otherwise indicated: three types of new azaphenothiazines with acetone−aqueous TRIS buffer as the mobile phase (R_M values were linearly dependent on the concentration of acetone and when extrapolated to 0% acetone gave the lipophilicity parameters R_{Mo} and log P_{TLC} (*34*); derivatives of 1,2,4-triazole and thiosemicarbazide with potential antituberculosis activity using mixtures of MeOH, ACN, and water as mobile phases (*35*); isomeric chalcones and cyclic chalcone analogues on bonded C-2 silanized silica gel layers with MeOH−water (6:4) mobile phase (*36*); novel diquinthiazines with mixtures of acetone and aqueous TRIS buffer, pH 7.4, as mobile phases (*37*); 2,6-disubstituted 7-methylpurines with mixtures of acetone and sodium acetate−veronal buffer, pH 7, as mobile phases (theoretical calculated and TLC lipophilicity values were compared) (*38*); new oxadiazoline derivatives with MeOH−water mobile phases (*39*); seven cortisone derivatives on silica gel 60 impregnated with paraffin oil and MeOH−water (6:4 and 7:3) mobile phases (*40*); and a series of potential antituberculosis drugs from a group of substituted anilides of pyrazine-2-carboxylic acid on C-2 bonded silica gel F plates impregnated with silicone oil and developed with 0.05 M phosphate buffer(pH 3.0 or 7.4)−methanol mobile phases (*41*). Additional studies of lipophilicity are cited in other sections of this review.

To improve the precision of R_f values and obtain thermodynamic values, the use of the following equation was recommended for silica gel TLC:

$$R_{st} = \frac{(R_f)_i}{(R_f)_{st}} = \frac{X_i}{X_{st}}$$

where X is the migration distance and the subscript i refers to the compound of interest and st to an unretained low molecular weight compound for which all sorbent pores are accessible, i.e., an analogue of the void volume marker in column chromatography. The model system for the study included phenacetin, acetanilide, meso-tetraphenylporphyrin, fullerene C_{60}, anthracene,

and three dyes as samples; silica gel F aluminum foil backed HPTLC layers; ascending development in an N-chamber saturated and unsaturated and S-chamber unsaturated; and toluene, diethyl ether, or THF−cyclohexane mobile phases (*42*).

Two new independent coefficients, R_U (retention uniformity) and R_D (retention distance), were proposed and their mathematical properties tested in wide range by Monte Carlo simulations. Their use for automatic screening and ranking of 114 TLC systems (silica gel, diol, or CN layers with hexane-modifier mobile phase and C-18 plates with pH 7.6 phosphate buffer-modifier mobile phase) in order of best R_f values was demonstrated in the separation of fibrate type antihyperlipidemic drugs by NP- and RP-TLC (*43*).

CHROMATOGRAPHIC SYSTEMS

Stationary Phases. TLC offers a greater variety of stationary phases than any other kind of chromatography to provide the required selectivity for a particular separation, including inorganic, organic, adsorption, partition, ion exchange, chiral, mechanically impregnated, polar and nonpolar chemically bonded phase, buffered, mixed, and gradient layers. However, the great majority of reported separations and analyses continue to be carried out in the adsorption mode using commercial, precoated NP silica TLC and HPTLC layers. This section will selectively review papers reporting fundamental studies on unmodified silica gel layers and analyses using layers other than silica gel. Additional diverse stationary phase/mobile phase systems are described in other sections of this review, especially the preceding one on Theory and Fundamental Studies.

The real water content, and therefore the activity, of a silica gel layer can be determined by applying a solution of Reichardt's dye (Dimroth's salt), measuring the absorption at 500 nm with a diode array scanner, and comparing with a calibration plot of peak height versus relative humidity (*44*). Circular dichroism and UV absorption spectra of silica gel used for coating commercial TLC plates provided convincing evidence that the layers are characterized by crystalline asymmetry of the silicon dioxide particle (*45*). A comparative study using a multicomponent analgesic tablet in the fluorescence quenching mode and a dye mixture in the visible mode showed that silica gel TLC plates often gave the best theoretical plate number (N) and resolution (R) values, but that development time, sensitivity, and linearity were better for HPTLC plates; spherical particle HPTLC plates performed better for the drug mixture and irregular particle HPTLC plates were better for the dye mixture (*46*).

Ultrathin layer chromatography (UTLC) plates, with a 10 μm thick monolithic layer grafted onto the glass plate with no binder, were successfully used as a rapid means of chromatographic separation prior to desorption electrospray ionization (DESI)-MS; increased speed of analysis compared with the use of standard surfaces was associated with the drying of solution phase samples (*47*). The rapid analysis of benzodiazepines in urine at the picomole level was shown to be feasible by combining two-dimensional (2D) UTLC separation followed by atmospheric pressure matrix assisted laser desorption/ionization MS (AP-MALDI-MS) (*48*).

Separations of alcohols, higher fatty acids, amino acids, and medicines were shown to be improved by impregnation of nonpolar (RP-2, RP-8, and RP-18) and polar [diol, CN, and amino (NH_2)] chemically bonded layers with solutions of squalene, L-lysine, L-arginine, sodium dodecylsulfate, L-(+)-tartaric acid, and D-(+)-galactose (*49*). Separations of 18 phenolic acids were reported on aminopropyl bonded silica with mobile phases comprising mixtures of diisopropyl ether and acetic acid with toluene, petroleum ether, or heptane (*50*). The best systems for separations of fluoroquinolone antibiotics were silica gel developed with MeOH−acetone−1 M citric acid−triethylamine (2.8:2:0.2:0.5) mobile phase, cellulose with dichloromethane−isopropanol−THF−25% ammonia (4:6:3:3), and C-18 bonded silica with MeOH−0.07 M phosphate buffer, pH 6−0.1 M benzyldimethyltetradecylammonium chloride (6:3:1) (*51*). Catechol type siderophores could be separated more effectively on polyamide layers than on silica gel (*52*).

In planar electrochromatography (PEC), an electrical field is applied across a TLC plate, and the resulting electroosmotic flow drives the mobile phase instead of capillary action as in normal TLC. A new plate for the method, prepared by fusing a mixture of silica gel and glass powder and then reaction with octadecyltrichlorosilane, was shown to have good mechanical stability and could be regenerated by soaking the chromatogram in acetone. Its use was shown by the separation of five dyes in a PEC chamber with a 2 kV intermittent voltage supply and aqueous ACN containing 25 mM sodium acetate buffer, pH 5, as the mobile phase (*53*).

The separation properties of five unconventional TLC phases, rice starch, microcrystalline cellulose, aminoplast, talc, and paraffin oil−impregnated silica gel, with mobile phases composed of water−methyl ethyl ketone−dioxane were evaluated in two studies using aliphatic linear alcohols with 1−20 carbon atoms as the esters of 3,5-dinitrobenzoic acid for test compounds. Separation factor (α), R, and Δ-R_f values were used to characterize the layers in the first study (*54*) and principal components analysis in the second (*55*).

A new "smart TLC plate" that integrates a linear array of amorphous silicon photodiodes was described (*56*). It consists of a standard TLC plate that was coupled to a 1 mm thick glass on which a 16 sensor array was fabricated and was developed in a small volume, horizontal PTFE (Teflon) chamber designed to house the plate and sensor substrate with optimum optical connection between them. Fluorescence of the test compound fluorescein was measured under UV light. It was demonstrated that the plate allows a TLC separation that can be observed in real time and terminated at the point of best resolution. When 2 μL each of 0.6 and 1.2 mg/mL fluorescein was spotted and developed, peak height was greater for the more concentrated solution, indicating the potential to obtain quantitative information on the plate. The concept of the technology seems to have promise, but for practical value much more development and research are needed to prove the ability to quantitatively analyze a wide range of different analytes and samples with low detection limits over all tracks of a plate.

The following analyses were reported on inorganic salt layers: densitometric quantification of cadmium in binary mixtures on a titanium silicate ion exchanger with detection using a saturated aqueous solution of sodium sulfide or mixed solution of 2,2′-bipyridyl and ferrous sulfate (*57*); separations of performance enhancing drugs on thin layers of bismuth silicate anion ex-

changer with organic, aqueous, and mixed organic aqueous−organic mobile phases (*58*); and separation of underivatized aliphatic polyamines (ornithine, citrulline, cadaverine, spermine, and spermidine) from urine on calcium sulfate layers with MeOH mobile phase followed by quantification by scraping of zones, elution of analytes, and spectrometry at 550 nm (*59*). Stannic silicate ion exchange layers were used for quantification of *p*-hydroxybenzoic acid, a toxic food contaminant, in the presence of preservatives with *n*-hexane−methyl ethyl ketone−acetic acid (8:2:0.3) mobile phase and scanning densitometry at 270 nm (limit of detection, LOD, and limit of quantification, LOQ, were 0.05 and 0.51 μg/zone, respectively) (*60*), and of the food preservative methylparaben in the presence of other food additives with the same mobile phase and scanning at 260 nm (LOD 0.29 μg/zone and LOQ 0.50 μg/zone) (*61*).

Mobile Phases. Mobile phases are usually chosen after searching the literature and guided trial and error testing of reported solutions with appropriate strengths (R_f values of 0.2−0.8 are optimum) and selectivities relative to the mixture to be separated. In addition, various systematic optimization procedures have been proposed. For example, two papers reporting the automatic selection of mobile phases for silica TLC using LSChrom software incorporating the Snyder theory were in the special issues of the *Journal of Liquid Chromatography & Related Technologies* mentioned in the introduction of this paper. A ternary mobile phase for the separation and identification of seven amino acids on microcrystalline cellulose, with detection by ninhydrin reagent, was optimized by use of the experimental design software packages Design-Expert 6 and Statistica; both products predicted the optimum mobile phase was butanol−glacial acetic acid−water (60.14:18.77:21.09), and this was proven experimentally (*62*). The best mobile phase for the analysis of five androstane isomers in drug formulations, biological samples, and natural sources was chosen from seven systems presented in the literature using the numerical taxonomy method, and it was found to be a mixture of chloroform, acetone, and petroleum ether; the proportions of these solvents were optimized by the simplex and Prisma methods (*63*). The separation of the ions of 12 metals on silica gel H (containing a small amount of colloidal silica gel but no other binder) was most successful when a mobile phase with a mixture of the ligands di(*n*-butyl)dithiophosphoric acid and trioctylphosphine oxide was used (*64*). MeOH−water mobile phase with sodium iodide additive gave highly correlated RP-TLC lipophilicity values for 11 new 1,2,4-triazole derivatives; the results obtained were discussed in connection with the known physicochemical properties of the seven monovalent anions that were compared for addition to the mobile phase (*65*). The fat soluble vitamins A and E and water soluble vitamins B-1, B-2, B-6, and B-12 were separated by silica gel HPTLC using fractional elution: benzene was the first mobile phase and a 0.02 M aqueous micellar solution of sodium dodecyl sulfate the second (*66*).

The following new systems for chiral separations were reported in the journal literature: molecularly imprinted polymers of L-mandelic acid (MDA) and its derivatives L-2-MDA and L-4-MDA as stationary phases with ACN−acetic acid (95:5) mobile phase for separation of racemates of the MDA series compounds (*67*); silica gel G (gypsum binder) F plates developed with phenol−water (3:1) and RP-18WF plates developed with mobile phases containing ACN and triethylamine−phosphate buffer (50 mM, pH 5.5) for separation of 17 DL amino acids after derivatization with Marfey's reagent and its chiral variants (*68*); RP-18F plates developed with the chiral mobile phase β-cyclodextrin−MeOH (15:1) for determination of *S*(+) and *R*(−) ibuprofen in drugs with UV densitometric detection at 222 nm (*69*); Macherey−Nagel ligand exchange Chiralplates developed with ACN−MeOH−aqueous phosphate buffer (pH 3−4 or 6−7) in a horizontal sandwich DS chamber for separation of amino acid enantiomers with detection by use of ninhydrin reagent and scanning at 518 nm with a diode array detector (DAD) densitometer (*70*); and silica gel G layers impregnated with a Cu(II)−arginine complex and developed with mixtures of ACN, MeOH, and water for resolution of some racemic β-adrenergic blocking agents (*71*). The number of papers published each year on chiral TLC is smaller than is deserved considering the potential value of the method, and it is hoped that the new book mentioned earlier (*11*), which contains a large amount of information on layers and mobile phases for separations and analysis of enantiomers and diastereoisomers, will lead to an increase in research and practical use in the field.

Additional stationary and mobile phase combinations are specified for separations reported in following sections of this review.

APPARATUS AND TECHNIQUES

Sample Preparation. An important difference between TLC and HPLC is that in TLC the sample is developed on a layer until the mobile phase reaches a premarked distance at the top of the layer, while in HPLC the sample is eluted until the slowest moving analyte leaves the column and is detected. The complete sample is contained in the thin layer chromatogram, and the plate is not reused. Strongly retained sample components may not be eluted from the column in an HPLC run, and they may elute later and interfere with subsequently injected samples on the same column. Therefore, sample purification (cleanup) is more critical in HPLC compared to TLC. Techniques for sample preparation prior to TLC were described in an encyclopedia article (*72*).

The following new solid phase extraction (SPE) methods were reported in the review period: extraction of veterinary drugs from wastewater using Strata-X cartridges prior to analysis on CN modified silica gel with 0.05 M $H_2C_2O_4$−MeOH (81:19) mobile phase with videodensitometric quantification (*73*); extraction on C-18 cartridges for quality control of *Rhodiola rosea* root extracts [silica gel layer, ethyl acetate−MeOH−water (77:13:10) mobile phase, UV slit scanning densitometry] (*74*); extraction of sulfonamides and trimethoprim from water on Waters Oasis copolymer hydrophobic−lipophilic balanced (HLB) cartridges [silica gel layer, chloroform−MeOH (89:11) mobile phase, videodensitometric quantification] (*75*); and extraction of enrofloxacin, oxytetracycline, and trimethoprim from wastewater on polystyrene−divinylbenzene (SDB) Empore disks [HPTLC CNF layer, 0.05 M $H_2C_2O_4$−MeOH (65:35) mobile phase, videodensitometric UV quantification] (*76*).

Ultrasonic extraction of anionic, cationic, and nonionic surfactants followed by TLC and videodensitometry was found to be much more efficient, faster, and less expensive for their determination in laundry wastewater samples compared to traditional shake-flask extraction and UV−visible (UV−vis) spectrometry (*77*). Antioxidant and antimycobacterial activities of *Tabernae-*

montana catharinensis were determined by TLC analysis of extracts obtained using supercritical fluid extraction (SFE) with carbon dioxide plus an alcohol or water cosolvent (*78*). Amino acids were separated by NP- and RP-TLC after formation of phenyl thiocarbamyl derivatives on the layer origin or in a tube prior to application; the iodine−azide reaction was used as the detection method (*79*).

Thin Layer Chromatography. Manual and instrumental methods for application of standard and sample solutions to plates were described in another new encyclopedia chapter (*80*). A device constructed for the drying of layers and postchromatographic derivatization detection of zones reduced uncontrolled propagation and nonhomogeneous vertical in-depth distribution of zones and improved precision of quantitative TLC by reflectance densitometry (*81*).

Microemulsion TLC (ME-TLC), in which a microemulsion is used as the mobile phase, was applied for fingerprinting of aqueous extracts of licorice (*Glycyrrhiza* spp.); it was shown that the ME-TLC is easier to operate, has better resolution and reproducibility, and has significantly different separation mechanism and retention behavior compared to conventional TLC (*82*). The chromatographic behavior of five macrolide antibiotics was studied by salting out TLC with a cellulose layer and aqueous ammonium sulfate solutions of different concentration as mobile phases; hydrophobicity was determined by linear relationships between compound R_M values and the ammonium sulfate concentration (*83*).

The great majority of TLC analyses are carried out by capillary flow ascending development in a large volume chamber (N-chamber) at ambient laboratory temperature. However, horizontal development in the Chromdes DS chamber is quite often reported in the literature. Suitability of this chamber placed in a circulating air incubator for separation of steroids by isothermal TLC at subambient and elevated temperatures on glass- and aluminum-backed layers was demonstrated, and practical aspects of the system were reported (*84*). A new variant of TLC was proposed in which the acidity of the mobile phase is gradually changed during development as a result of its contact with an acidic or basic gas phase that replaces the initial mobile phase vapor in the chamber; the potential of this approach was shown by using carbon dioxide and ammonia gases to improve the resolution of benzoic acids and aromatic amines on polyamide plates (*85*). A new type of TLC was described that involved covering an aluminum-backed silica gel foil with Parafilm M polymeric film, except for the bottom portion where it dipped into the mobile phase, and sandwiching it between two glass plates; testing with mixtures of dyes and B vitamins showed faster development times but poorer efficiency compared to classical TLC (*86*).

A large zone capacity for complex mixtures can be achieved by 2D (or bidimensional) TLC, in which a sample is spotted at the corner of a layer that is developed in orthogonal directions with two mobile phases having complementary separation mechanisms (with drying between developments), or by the use of two layers with complementary mechanisms. Spot and mobile phase anomalies in displacement mode TLC and 2D TLC were studied, and advantages and variations of the methods were described (*87*). Closely related coumarins from plant extracts of the *Apiaceae* family were separated by 2D TLC on cyano and diol bonded silica gel layers using nonaqueous mobile phases to obtain the adsorption mode and aqueous mobile phases at 90 ° for the partition mode; Whatman multiphase plates with a C-18 strip adjacent to a silica gel layer were also used with appropriate RP- and NP-mobile phases in each direction (*88*). Phenolic compounds were separated by 2D TLC on connected diol or silica gel for NP-TLC and RP-18W for RP-TLC; the plates were connected with 2 mm overlap by a special device (*89*). 2D-TLC and multimodal separations combining TLC with another separation method such as HPLC have great promise for increasing sample resolution, and more research on these techniques is expected in the future.

Multiple development is another method for improving resolution in capillary flow TLC. Amino acids were separated on cellulose layers by manual multiple development in a Chromdes DS chamber; eight 10 mm developments with ACN−water (8:2) or isopropanol−water−acetic acid (89.5:9.5:1) were used, with layer drying between runs (*90*). Automated multiple development (AMD) is carried out in a Camag chamber with increasing development lengths and an isocratic mobile phase or a mobile phase gradient with decreasing strength. Reported applications include the stability indicating HPTLC assay of leuprolide acetate [silica gel 60 layer, five step incremental multiple development with ethyl acetate−MeOH−25% aqueous ammonia (6:3:1), densitometry at 280 nm] (*91*); analysis of some fibrate-type antihyperlipidemic drugs (diol F_{254} plates, THF−hexane mobile phase, densitometry at 245 and 277 nm) (*92*); analysis of ginsenosides from *Panax quinquefolium* L (silica gel $60F_{254}$, MeOH−chloroform gradients, detection with Godin's reagent, densitometry at 540 nm) (*93*); and separation and quantification of terpenoids of *Boswellia serrata* Roxb. (HPTLC silica gel F layer; mobile phase gradients; densitometry at 254 nm or 560 nm after derivatization with anisaldehyde−sulfuric acid reagent) (*94*).

OPLC (overpressured layer chromatography or optimum performance laminar chromatography) is a forced flow method performed in an instrument (OPLC-NIT Co.) in which the plate containing an optimized HP layer is covered with a flexible, inert polymer sheet under pressure and mobile phase is pumped through the sorbent layer. In effect, the layer is analogous to a flat HPLC column. OPLC was combined with UV densitometry and near-infrared (NIR) spectrometry directly on the layer for qualitative and quantitative pharmaceutical analysis (*95*). Other published applications of OPLC included a complete multidimensional separation of a tetrazine library prepared by parallel combinatorial synthesis obtained using 2D OPLC (*96*), biochemical examination of the general defense mechanism of plants (*97*), and separation and quantitative determination of seven aldoses and alditols in hemicellulose hydrolyzates [aluminum foil backed OPLC-HPTLC plates, ACN−acetic acid−water (63:33:5) mobile phase, overrunning elution] (*98*).

A novel strategy was presented for fractionation of a complex peptide mixture using 2D PEC/TLC coupled with MALDI-TOF MS and tandem MS (MS/MS); phosphopeptides migrate more slowly in the first dimension, based on their anionic phosphate residues, and predominantly acidic phosphopeptides migrate in the opposite direction relative to the bulk of peptides, while phosphopeptides are further distinguished based on hydrophobicity in the second dimension (*99*). High pressure PEC (PPEC) instruments were shown to give a linear voltage drop and constant

electroosmotic flow (EOF), in contrast to low pressure (or no applied pressure) PEC; plate equilibration times, increased field strengths, sample application designs, and use of rhodamine B as a visual marker for reproducible migration and calculation of *N* values were studied for PPEC as an emerging technique for obtaining higher separation efficiency for TLC (*100*). A new device was described and for performing PPEC in which the whole area of the plate is pressurized and mobile phase flow is directly controlled; the effects of various instrumental operating parameters on separation efficiency were evaluated (*101*). PPEC using an adsorbent layer closed with a polymer film was described (*102*). Continuing research on electrodriven TLC is expected in coming years, but wide acceptance for practical analyses has not yet occurred.

The DryLab 2000 plus HPLC simulation software was used to simulate TLC separations and transfer them to HPLC columns based on data from preliminary TLC separations. For data entry, R_f values from TLC were converted to retention times, development distance on the plate was used as column length, and layer thickness was used as the column diameter. Three preliminary runs were needed to simulate TLC separations, and to produce a reasonably accurate HPLC simulation based on TLC data, it was found that a higher number of preliminary experiments was required (*103*).

DETECTION AND IDENTIFICATION OF SEPARATED ZONES

Zone detection in TLC is based on natural color, fluorescence, or UV absorption (fluorescence quenching on phosphor-impregnated or F layers) or on the use of various universal or selective chemical or biological detection reagents applied by spraying or dipping. Densitometric scanning in fluorescence and absorbance modes is an important detection and documentation technique, as well as for quantitative analysis. A great advantage of TLC lies in the ability to use a number of detection methods and reagents in sequence on a single layer storing the entire sample in the chromatogram to increase the amount of information obtained. Identification is initially based on the correspondence of R_f values and detection characteristics between sample and standard zones but must be confirmed by other evidence, such as offline or online coupling of TLC with various spectrometric methods. The ability to apply multiple detection methods without the time constraints of column elution chromatography, the need for less sample cleanup, and the high throughput and resultant speed and economy offered by the simultaneous analysis of many samples on a single layer with appropriate standards will almost certainly lead to a wider use of TLC and HPTLC in the future.

Additional detection and identification methods are cited in other sections of this review.

Chemical Detection. Heating at 60 °C for 15 min or more, rather than the usual shorter heating time at a higher temperature, was found to give the best results for detection and quantification of cholesterol and bile aids on silica gel and RP-18W layers after application of phosphomolybdic acid (PMA) reagent (*104*). 2,3-Dichloro-1,4-napthaquinone followed by isatin were introduced as new reagents that produce distinguishable and stable (24−48 h) colors with many amino acids on silica gel G plates with a detection limit between 0.01 and 0.3 μg (*105*). Bromocresol green, bromophenol blue, potassium permanganate, methyl red, and alkaline blue were found to be the best reagents for detection of short chain fatty acids and their ammonium salts separated on silica gel (*106*). Detection with the iodine−azide reaction was described for amphetamine and its analogues (*107*), mercaptopyridines and mercaptopyrimidines (detection limit 1−20 pmol for HPTLC and 1−60 pmol for TLC) (*108*), and heterocyclic thiols (e.g., 2-thioguanine and 6-mercaptopurine) in biological samples (*109*).

The general contribution of nonspecific interactions to fluorescence intensity was studied for TLC and HPLC, and it was found that the nature and concentration of the probe (reagent) determine response and detection sensitivity for a given sample (*110*). Coralyne cation was found to be a more sensitive fluorescent probe for general detection compared to the previously described general probes berberine cation and Reichardt's dye; the method was demonstrated by fluorescence scanning densitometry on HPTLC plates impregnated with a solution of coralyne cations for analytes such as longchain alkanes, alcohols, alkylbromides, and neutral lipids (*111*). Spraying with sodium hypochlorite, hydrogen peroxide, or potassium hexacyanoferrate(III)−sodium hydroxide reagent gave vivid blue fluorescent zones under 365 nm UV light at 0.01−0.06 μg levels for tryptophan, serotonin, and psychoactive tryptamines (*112*).

Heating of amino HPTLC plates to produce fluorescent zones of certain compounds viewed under 366 nm UV light is a useful detection method. This thermochemical reagent free derivatization method was used in the determinations of the mycotoxin sterigmatocystin at a level of 2 μg/kg in cereal grains (*113*) and sucralose in a milk based confection with an LOD of 6 ng/band (*114*). It had been shown earlier (*115*) that this reagent free detection method was not limited to amino plates and fluorescent zones when simple heating of a silica gel F plate allowed detection of creatine zones by fluorescence quenching under 254 nm UV light.

Biological Detection. A novel TLC based method by which cellular phosphoinositides are separated, transferred, and detected by specific phosphoinositide binding domains (TLC-blotting) was developed for use in physiological studies and for diagnosing metabolic disease and cancer (*116*). TLC-bioautography was applied in the following investigations: screening of enrofloxacin and ciprofloxacin residues in milk with isolation and concentration by matrix solid phase dispersion (MSPD) (*117*); detection of xanthine oxidase inhibitors and superoxide scavengers (*118*) and glucosidase inhibitors (*119*) in complex matrixes; investigation of *Chelidonium* alkaloids using the BioArena system integrating TLC and/or OPLC with bioautography (*120*); and validation of a method for determining spiramycin, virginiamycin, and tylosin in feed samples (*121*).

The potential for increased use of biological methods for sensitive and selective TLC detections is very great, and an increasing number of papers in this area are to be anticipated in future years.

Thin Layer Chromatography/Mass Spectrometry. A continuing very high level of research in the techniques and applications of TLC coupled with MS is one of the major trends in the past 2 years, and it is anticipated that TLC/MS method development efforts will increase in the future.

Cholesteryl ester hyperoxide isomers were detected in biological samples by using gas chromatography (GC)/electron ionization-MS following TLC blotting with diphenyl-1-prenylphosphine (DPPP) fluorescence detection offline; the DPPP derived fluorescent spots were extracted and derivatized to methyl ester/trimethylsilylether derivatives for GC/MS (*122*). Alkaloids in goldenseal and dietary supplements were qualitatively and quantitatively analyzed directly from NP-TLC plates by DESI-ion trap MS (*123*) with automated sampling and imaging (*124*, *125*). Combined preparative HPTLC immunostaining overlay assay and nano-ESI quadrupole TOF MS/MS was applied to the structural characterization of high-affinity and low-affinity binding ligands of Shiga toxin I and has high potential for a broad range of other medical and chemical applications (*126*).

TLC coupled online with TOF-secondary ion mass spectrometry (TOF-SIMS) gave successful analyses of organic halides (negative ion MS) and amines (positive ion MS) on silica gel (*127*) and of diterpenes on silica gel UTLC layers and aluminum-backed silica gel using positive and negative microscans (*128*).

Morlock and co-workers published a series of papers on TLC/MS applied to pharmaceutical and food samples. ITX was quantified in milk, yogurt, margarine, and soy bean oil by accelerated solvent extraction (ASE), HPTLC on silica gel 60 [toluene−*n*-hexane (9:1) mobile phase] and RP-18 [ACN−water (9:1) mobile phase] layers, and fluorescence densitometry (254/>400 nm); positive results were confirmed by ESI-SIM/MS and DART-MS (*129*). A plunger based extraction interface (now commercially available as the ChromXtract) used with an HPLC pump was shown to provide good results for quantitative TLC/ESI-MS on HPTLC silica gel plates in terms of repeatability of the MS signal, LOD, and LOQ for the model compound Harman, a heterocyclic aromatic amine (*130*). Modifications of the device enabled complete, consistent extraction of analytes from glass backed as well as aluminum backed TLC and HPTLC plates, layers with thickness up to 100 μm, and different stationary phases (*131*). The ChromXtract was used in the simultaneous determination of caffeine, ergotamine, and metamizole in a solid pharmaceutical formulation by HPTLC [silica gel 60F layer, ethyl acetate−MeOH−ammonia (90:15:1) mobile phase] with UV absorption densitometry at 274 nm for caffeine and metamizole and fluorescence densitometry at 313/>340 nm for ergotamine; mass confirmation was by single quadrupole MS in the positive ESI full scan mode for caffeine and ergotamine and negative mode for metamizol (*132*). A new HPTLC/DART-TOF MS coupling method was shown to be successful for identification and qualitative purposes with a detection in the low ng/zone range for ITX; quantitative analysis was improved by using isotope labeled standards, and spatial resolution by use of an in-house plate holder system was better than 3 mm (*133*). A new HPTLC/ESI-MS method for quantification of caffeine in pharmaceutical and energy drink samples used stable isotope dilution analysis (SIDA); after HPTLC on a silica gel 60F layer with overspotting of samples and caffeine standard with caffeine-d_3 used for correction of the ChromXtractor plunger positioning, densitometric detection was carried out a 274 nm followed by elution of bands into a single quadrupole mass spectrometer (*134*).

The following new directly coupled TLC/MALDI-TOF MS analyses were reported: peptides using PEC and TLC separations to achieve separation by both electrophoretic and capillary forces, labeling of separated analytes with a fluorescent dye and visualization with a Xe-arc lamp based-CCD (charge coupled device) camera system, and direct orthogonal MALDI-TOF MS with the matrix spotted on top of the fluorescence imaged spots (*135*); neutral glycosphingolipids by TLC-immunostaining and MALDI quadrupole ion trap (QIT) TOF MS/MS (*136*); polyethylene glycol, polypropylene glycol, and polytetramethylene glycol mixtures (*137*); phospholipids from egg yolk (*138*); primary amine end-functionalized polystyrene and poly(methyl methacrylate) synthesized by living anionic polymerization techniques (*139*); peptides and proteins after separation on a layer of porous polymer monolith [50−200 μm poly(butyl methacrylate-*co*-ethylene dimethacrylate)] (*140*); and human and elephant milk oligosaccharides using silica gel layers, glycerol liquid matrix, an infrared (IR) laser for volume material ablation, soft desorption/ionization conditions, and an orthogonal TOF mass spectrometer (*141*). The European Pharmacopeia (5th ed.) method for the four principal triterpenoid compounds of *Centella asiatica* was modified to include silica gel plates, ethyl acetate−MeOH mobile phase, anisaldehyde detection reagent, and MALDI-TOF MS separated zone confirmation (*142*).

TLC with an aluminum backed silica gel sheet developed with benzene−hexane−chloroform (1:1:1 or 5), UV detection, and offline laser TOF MS identified hydroxylamino-dinitrotoluenes, unstable products from 2,4,6-trinitrotoluene metabolites formed by bacteria (*143*) and spontaneous conversion products of the unstable metabolites (*144*). A rapid screening method for arsenic speciation in biological samples was based on silica gel TLC with acetone−acetic acid−water (2:1:1) mobile phase and laser ablation inductively coupled plasma (ICP)-dynamic reaction cell-MS; the LOD ranged from low picogram to nanogram levels (*145*).

GC/MS was used to study the products cleaved from RP-18 chemically bonded layers during heating at 165 °C. Compounds were extracted by accelerated solvent extraction, and alkenes and carbonyls were identified; aromatic products remained uncleaved. This study sheds light on the mechanism of layer thermal modification and, indirectly, about its unmodified surface properties (*146*).

Thin Layer Chromatography Coupled with Other Spectrometric Methods. Organic reaction products could be identified directly from separated TLC spots by high-resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) spectrometry. The spot is scraped from the plate, transferred to an HRMAS sample rotor, and suspended with deuterated solvent. The broad background signal near 4.6 ppm caused by the presence of the stationary phase could be removed by using either a Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence or chemical exchange, and spectra with a good signal-to-noise ratio (S/N) were obtained in a few minutes for low analyte amounts (*147*).

Cellulose TLC with *n*-butanol−acetic acid−water (8:4:2) mobile phase was hyphenated to near IR (NIR) spectrometry for the qualitative and quantitative analysis of apple juice for L-lysine and other amino acid determinations in crude samples. The LOD was 0.01 mg/mL (*148*).

The ingredients in *Evodia rutaecarpa* Chinese traditional medicine were analyzed at the microgram level by HPTLC and

surface enhanced Raman spectrometry (SERS). No standard samples were needed (*149*).

QUANTITATIVE ANALYSIS

State of the art quantification in TLC and HPTLC involves the following steps: sample preparation to remove interfering impurities but retain essentially all of the analyte; application of a series of standard reference solution aliquots and bracketed sample aliquots to the layer as spots or bands; development of the layer; detection of separated analyte zones; measurement of zone areas with a densitometer (slit scanning, diode array, video, or digital camera or flatbed scanner based instrument); generation of a calibration graph by linear or polynomial regression of the areas and weights of the standards; interpolation of the weights of sample zones from the graph; calculation of the concentration of the analyte in the sample; and validation of the results. The excellent results of quantitative HPTLC are becoming increasing apreciated and will lead to the greater use of densitometric analysis for a wider range of applications in the future.

Techniques and Instruments. The ability of the Kubelka−Munk theory to correctly describe TLC evaluations was studied. Uniform drying of all tracks on the layer leading to constant sample distribution in each spot was found to be critical. The theory was modified and extended to describe isotropic as well as asymmetrical scattering and to include a formula for absorption and fluorescence in diode array densitometry (*150*).

Real time acquisition of images of spots on TLC plates during development was studied quantitatively. Procedures were described for imaging using a CCD camera and for image processing, incorporating corrections for fixed pattern effects and compensation for the moving solvent front, to measure the absorbance of the analyte. Imaging of Sudan II was carried out in the transmission mode, and peak areas were found to be time independent. Quantification of the relationship between peak area and sample loading was established over the range of 1−50 ng (*151*).

Seven veterinary pharmaceuticals were determined in production wastewater by SPE on hydrophilic−lipophilic balanced cartridges, HPTLC on a CN modified layer with $H_2C_2O_4$−MeOH (81:19), and videodensitometry at 254 and 366 nm (*152*).

A method was presented to reconstruct the nonuniform background for camera based quantitative evaluation of TLC. The concave feature of illumination produced by a linear light source on a plane and a convex hull algorithm are used to find points belonging to the background. Then, B-spline is employed to reconstruct the background. It was found experimentally that the correlation coefficient between the linear samples is 0.9944 after removing the estimated background (*153*). The same author found that the main limitation in CCD quantitative evaluation of TLC is its nonuniform illumination. To solve the problem, the background is estimated by employing the cubic spline surface construction, then the estimated background is subtracted from the original image to make the image segmentation. Finally, the gray level integration for each object is calculated, which is proportional to the value of the samples. The linear correlation coefficient between gray integration and concentration of samples was 0.9978 (*154*).

An SPE−TLC-fiber-optic scanning densitometric method was reported for determination of flavonol aglycones (quercetin and kaempferol) in extracts of rose leaves. C-18 SPE cartridges, silica gel, or cellulose layers, automatic sample application, development in a DS chamber, scanning with a diode array densitometer in the range 200−550 nm, and quantification based on peak area comparison with calibration plots were the conditions used. Compounds in the range of 1−10 μg/spot were identified by automatic measurement and computer based comparison of in situ UV spectra with reference spectra using a cross-correlation function (*155*).

The ChromImage flatbed scanner densitometer with Galaxie-TLC image analysis software was used successfully for quantitative silica gel HPTLC. The visible mode was evaluated by determination of the recovery of rhodamine B standard dye from a four dye mixture and determination of the precision of replicate analyses. Determinations of caffeine in multicomponent analgesic tablets and a cola beverage were performed in the fluorescence quenching mode on HPTLC plates with a brilliant UV indicator (Adamant) (*156*).

"Digitally enhanced TLC" (DE-TLC) was introduced as an inexpensive, new technique for qualitative and quantitative analysis that can be used in any laboratory, but especially high schools and colleges, that cannot afford a commercial densitometer. The DE-TLC equipment consisted of a 254 nm UV lamp on a stand, Nikon D 1 × 6 megapixel digital camera, and free software. Digital images, multispectral scans, densitograms, calibration curves, and analytical results for model compounds, including luminol, riboflavin, nicotinamide, and vanillin, were described (*157*).

A great strength of TLC is the ability to analyze organic compounds without chromophores by application of detection reagents (often called staining reagents). The use of staining reagents such as anisaldehyde, vanillin, and sulfuric acid in combination with image analysis software (JustTLC) was shown to give reliable results, suitable for high throughput screening or physical organic investigations. Errors due to the staining process can be diminished by measuring ratios of compounds (*158*).

Quantitative methods must be validated to prove the reliability of the obtained results. Factors considered may include accuracy (recovery), precision [repeatability and intermediate precision in terms of relative standard deviation (RSD)], specificity, linearity, range, limits of detection and quantification, and robustness. The International Conference on Harmonization (ICH) developed guidelines for validation of pharmaceutical analyses based on characteristics such as these, and most published TLC analyses use all or part of the ICH model of requirements for quality assurance of results. Three papers by Morlock and co-workers cited in the previous section (*129*, *132*, *134*) and the following papers are examples of densitometric methods that were extensively validated: heterocyclic aromatic amines in meat [HPTLC silica gel layer preconditioned with ammonia vapor and developed with MeOH−chloroform (1:9) mobile phase] (*159*); simultaneous analysis of costunolide and dehydrocostus lactone in *Saussurea costus* (validated in accordance with ICH guidelines) (*160*); trigonelle in herbal extract and pharmaceutical dosage form [silica gel 60F, *n*-propanol−MeOH−water (4:1:4), absorbance scanning at 269 nm] (*161*); aceteoside in the leaves of *Plantago palmata* Hook f.s. [two general validation protocols were proposed for HPTLC with UV and visible (postchromatographic derivatization with sulfuric acid−vanillin reagent) detections] (*162*); and glu-

cosamine in an herbal dietary supplement [silica gel 60F, 2-propanol−ethyl acetate−8% ammonia solution (1:1:1), anisaldehyde detection reagent, reflectance scanning at 415 nm] (*163*).

Applications. In addition to the many quantitative analyses already cited in this review, this section presents selected, important examples of a variety of analytes and sample types for which densitometic methods have been reported in the past 2 years. Validation data are given for many of the applications to document the excellent quantitative results that are possible using modern TLC and HPTLC, and the major importance of TLC in pharmaceutical and herbal medicine and supplement analysis is illustrated by the chosen papers.

Oleanolic acid was quantified in *Oldenlandia corymbosaL.*L. whole plant powder on silica gel 60F layers with dichloromethane−toluene−acetone−MeOH (3:4:1.5:0.3) mobile phase. Zones were detected with Liebermann−Burchard reagent and scanned at 529 nm. LOD and LOQ were 0.1 and 0.5 μg, respectively, and the linear response range was 1−9 μg (*164*).

HPTLC determination of swertiamarin and amarogentin in *Swertia* species from the western Himalayas was performed on silica gel 60F plates with ethyl acetate−MeOH−water (77:8:8) mobile phase and densitometry at 235 nm. Average recoveries were 94.5 and 96.5%, respectively (*165*).

Iranian liquid products of *Matricaria chamomilla* L. were analyzed for apigenin by direct application to silica gel 60F plates, development with toluene−MeOH (10:2), and densitometry at 343 nm. The repeatability was 2.2% RSD, and the average recovery at two levels was 101% (*166*).

HPTLC silica gel 60F layers, toluene−acetone−MeOH (3:1:1) mobile phase, and scanning at 312 nm were applied to determination of sildenafil citrate in herbal medicinal formulations. Recoveries from powders and tablets were 83.2 and 99.4%, respectively, and the calibration curve was linear ($r = 0.9993$) from 100−600 ng/spot (*167*).

Berberine content in 80% aqueous ethanol stem macerates of *Coscinium fenestratum* was found by development of silica gel GF TLC plates with butanol−glacial acetic acid−water (14:3:4) and detection and quantification by densitometry at 415 nm. The linearity range was 240−840 ng, interday and intraday RSDs were <4.1%, recovery was 97.6−98.7% with an RSD of 3.8%, and LOD and LOQ were 20 and 50 ng/spot, respectively (*168*).

Quantification of valerenic acid in *jatamansi* and *Valeriana officinalis* involved use of silica gel 60F HPTLC aluminum backed plates, hexane−ethyl acetate−acetic acid (80:20:0.5) mobile phase, detection with anisaldehyde−sulfuric acid reagent, and absorption−reflectance mode densitometry at 700 nm. The calibration curve was linear from 500 ng−2.5 μg (*169*).

Phyllanthin and hypophyllanthin from *Phyllanthus* species were separated on silica gel 60F layers developed with hexane−acetone−ethyl acetate (74:12:8), visualized through color development with vanillin in concentrated sulfuric acid and ethanol, and quantified by scanning at 580 nm. Recoveries were 98.7 and 97.3%, respectively (*170*).

Ethanol extracts (15%) of *Senna siamea* leaves and flowers were analyzed for barakol on aluminum backed silica gel 60F TLC plates with chloroform−MeOH (85:15) solvent system and absorbance densitometry at 366 nm. Linearity was found over the range 200−900 ng/spot, RSD was <0.5%, accuracy averaged 101%, and LOD and LOQ were 8 and 50 ng, respectively (*171*).

An herbal medicinal product containing *Aesculus* and *Vitis* dry extracts in capsules was analyzed for aescin. After a purification step using C-18 SPE cartridges, samples were analyzed on a silica gel HPTLC plate with the upper phase of acetic acid−water−butanol (1:4:5) as the mobile phase. Spots were visualized by spraying with anisaldehyde reagent and heating, and they were measured by scanning at 535 nm. Validation according to ICH guidelines indicated the method had a standard graph with a linearity correlation of >0.99 from 0.16−0.80 μg/spot, recovery of 100%, and RSD with respect to time and concentration of 1.3 and 1.5%, respectively (*172*).

A rapid and simple method using RP-18F plates developed with 0.2% aqueous trifluoroacetic acid−ACN (35:65), derivatization of spots with anisaldehyde reagent, and absorption−reflectance scanning at 426 nm allowed simultaneous determination of artemisinin, artemisinic acid, and arteannuin-B in *Artemisia annua* (*173*).

Phyllanthin, hypophyllanthin, gallic acid, and ellagic acid were simultaneously quantified in *Phyllanthus amarus* by HPTLC on silica gel 60F plates with toluene−ethyl acetate−formic acid (6:2:1) mobile phase and scanning at 280 nm. UV absorption spectra were recorded in situ to confirm the identity of the spots. Validation of results for the four analytes was according to ICH guidelines: instrumental precision ranged from 0.08−0.93% RSD, repeatability of the method from 0.79−1.1% RSD, and average recovery at three different levels from 98.7−100.5% (*174*).

For quantification of xanthohumol in hops (*Humulus lupulus* L.) and hop products, MeOH ultrasonic extracts were separated on silica gel 60 HPTLC plates with toluene−dioxane−acetic acid (77:20:3) mobile phase and the chromatograms scanned at 368 nm. Intraday and interday RSD were 1.7 and 2.3%, respectively, and instrumental precision and repeatability were 0.38 and 1.5%, respectively. Accuracy averaged 104% at three levels (*175*).

Salicylamide was determined in diuretic and analgesic tablets containing caffeine, acetaminophen, potassium salicylate, and aspirin as additional ingredients. Within day precision averaged 0.80% and interday precision 1.3%. Accuracy ranged from 0.40−1.8% (*176*).

A comparison was made of HPTLC-densitometry and column HPLC for the determination of flavonol aglycones and terpenelactones in *Ginko biloba* extract. On the basis of accuracy and precision data, HPTLC was shown to be a time saving complement to HPLC for routine analysis (*177*).

TLC with multiple detection and confirmation by ESI-single quadrupole MS was used for analysis of energy drinks. After separation, plates were scanned by UV absorbance measurement at 261 nm for determination of nicotinamide and 275 nm for caffeine; by fluorescence measurement at 366/>400 and 313/>340 nm for riboflavin and pyridoxine, respectively; and by visible absorbance measurement at 525 nm for taurine after postchromatographic derivatization with ninhydrin reagent. Calibrations were linear or polynomial with $r^2 > 0.99$, overall recoveries of the five compounds were between 81 and 106% at three concentration levels, repeatabilities were between 0.8 and 1.5% RSD, and intermediate precisions ranged between 0.5 and 7.4% at different concentration levels (*178*).

A quantitative method using TLC plates with a concentrating zone and UV absorption densitometry was described for caffeine determination in human saliva and urine. Results corresponded with those obtained with GC, HPLC, radioimmunoassay, and enzyme immunoassay, but the TLC method was faster and less expensive (*179*).

Phospholipid and sphingolipid content in the feces of uninfected BALB/c mice and those infected with *Echinostoma caproni* was determined by silica gel HPTLC with a chloroform−MeOH−water (65:25:4) mobile phase, cupric sulfate detection reagent, and visible mode densitometry in a search for useful biological markers to distinguish infections in mice from uninfected controls (*180*).

TLC on silica gel preadsorbent plates showed that the concentrations of glucose and maltose were significantly reduced in *Biomphalaria glabrata* snails infected with *Schistosoma mansoni* as well as those uninfected when the snails were estivated. The carbohydrates were extracted from the digestive gland−gonad complex (DGG) of the snails with 70% aqueous ethanol, the mobile phase was ethyl acetate−glacial acetic acid−MeOH−water (60:15:15:10), zones were detected with α-naphthol−sulfuric acid reagent, and quantification was by visible mode densitometry (*181*).

Carbohydrates were determined during pharmaceutical fermentations on silica gel in a saturated twin trough chamber with chloroform−carbon tetrachloride−35% aqueous formic acid−MeOH (20:5:17:22). After the layer was dried, it was dipped in sulfuric acid reagent, and the detected spots were measured by absorbance densitometry at 290 nm (*182*).

The major triterpenoid saponins, bacoside A3 and bacopaside II, were determined in herbal extracts and commercial formulations. HPTLC RP-18F layers were developed with toluene−MeOH−ethyl acetate (15:5:4) in the dark in a controlled humidity chamber (55−65% humidity). Densitometry was performed at 344 nm (*183*).

Methionine was determined in pharmaceuticals after separation on silica gel in a presaturated chamber with *n*-propanol−water−chloroform (5:2:1). Quantification was based on the oxidation and reaction of methionine with leuco xylene cyanol FF solution, which formed a blue dye. Absorbance densitometry was at 613 nm. Separation from L-cystine, calcium pantothenate, vitamins B1 and B7, and *p*-aminobenzoic acid was reported (*184*).

Variations in the neutral lipid composition of *Helisoma trivolvis* and *Biomphalaria glabrata* snails caused by increased water salinity were measured by HPTLC of DGG chloroform−MeOH (2:1) extracts on preadsorbent silica gel HPTLC plates developed with petroleum ether−diethyl ether−glacial acetic acid (80:20:1). The detection reagent was 5% PMA in MeOH, and absorbance−reflectance scanning of the resulting blue lipid fraction zones was at 610 nm (*185*).

The effects of different diets on the β-carotene and lutein content of whole bodies and DGGs of the popular aquarium snail *Pomacea bridgesii* were determined by acetone extraction of the pigments, separation on preadsorbent C-18 HPTLC layers developed in the dark with petroleum ether−ACN−MeOH (1:1:2), and visible mode reflection−absorbance densitometry (*186*).

Andrographolide and 14-deoxy-11,12-didehydroandrographolide were quantified in *Andrographis paniculata* Nees to determine its antioxidant potential. Silica gel layers were developed with chloroform−MeOH (4:1) after 2 h chamber saturation to separate the analytes, and densitometry was at 254 nm. Linearity was obtained between 10 and 2000 μg/mL, LOD was 3.3 μg/mL, mean recoveries were >97%, and inter- and intra-assay variation between 0.86−0.98% (*187*).

A stability indicating method (alkaline, acidic, oxidative, thermal, and photodegradation) was successfully validated for alfuzosin hydrochloride in bulk powder and formulations. Silica gel layers were developed with MeOH−ammonia (125:3), and separated zones were scanned for absorption at 245 nm. The linearity of determination was between 0.5 and 7.0 μg, and the average recovery was 95.8−102% (*188*).

Gymnema sylvestre R. Br. could be standardized by reference with gymnemagenin by HPTLC. Silica gel was developed with chloroform−MeOH (9:1), and quantitative determination was by densitometric absorbance measurement at 290 nm. Linearity was observed in the 4−10 μg range, the average recovery from an extract was 99.1%, and the content of leaves was 1.61% (*189*).

The following are additional studies involving slit scanning densitometry in the absorbance−reflectance mode: determination of nitrendipine in tablets (scanning at 335 nm) (*190*); determination of lipids and phospholipids in the medicinal leech *Hirudo medicinalis* maintained on different diets (scanning at 610 and 370 nm) (*191*); detection of the iridoids of *Satchys* species growing in Hungary (detection with Erlich's spray reagent and scanning at 540 nm) (*192*); monitoring the dose of florfenicol in medicated salmon feed (scanning at 223 nm) (*193*); standardization of the Ayurvedic medicinal plant *Piper longum* (scanning at 260 nm) (*194*); evaluation of the stereochemically peculiar 2D separation of 2-arylpropionic acids by chiral TLC (concentration profiles recorded by scanning at 210 nm) (*195*); separation of indole alkaloids from *Rauvolfia serpentina* roots (detection by dipping in Dragendorff reagent and scanning at 520 nm) (*196*); quantification of pantoprazole sodium sesquihydrate and domperodone in tablets (scanning at 286 nm) (*197*); determination of itopride hydrochloride in a pharmaceutical preparation and bulk drug (scanning at 230 nm) (*198*); analysis and purification of synthesis products (scanning at 280 nm, AP-MALDI-MS, and PLC) (*199*); quantitative screening of the myricitrin content of crude methanolic extracts of *Acer* species leaves (scanning at 254 nm) (*200*); simultaneous determination of diclofenac sodium and paracetamol in a pharmaceutical preparation and bulk drug powder (scanning at 260 nm) (*201*); quantitative analysis of selected phenolic acids for standardization of *Propolis* concentrates (scanning at 320 nm) (*202*); quantification of phenobarbital in a dosage form (scanning at 210 nm) (*203*); monoterpenoid alkaloid assay in areal tissues of *Catharanthus roseus* (scanning at 280 nm) (*204*); and determination of curcuminoids from *Curcuma longa* (scanning at 366 nm) (*205*).

PREPARATIVE LAYER CHROMATOGRAPHY

As mentioned above, the first comprehensive book on the practical and theoretical aspects of capillary flow PLC was published (*10*). Microbrushes were proposed for reproducible sample application in linear and circular PLC (*206*).

A PLC procedure was described for purification of synthesis products. Samples were applied manually with a syringe as thin 1.5 cm rectangular bands 1.5 cm apart to a 2 mm thickness layer of silica gel 60F. Vertical development in a saturated chamber with chloroform or dichloromethane mobile phase required 60

min, after which the zones were detected under UV light and compounds recovered by scraping and elution using a device constructed from a Finntip 1000 and Bio-Inert Microsep II membrane. Purity of the products was established by analytical HPTLC−UV and atmospheric pressure (AP)-MALDI-MS (*207*).

Gradient PLC and RP-HPTLC were used for analysis of anthocyanin extracts from *Malve arborae* L. floss and *Vaccinium myrtillus* L. fructus. Compounds in the extracts were separated on silica gel 60F PLC plates by a five step gradient with mobile phases containing different amounts of methyl *tert*-butyl ether (MTBE) as modifier in a chamber with a mobile phase reservoir/injector. PLC was followed by rechromatography on RP-HPTLC plates with a mobile phase prepared from MeOH, water, and formic acid. Anthocyanin standards can be prepared by the method (*208*).

PLC was used in the isolation and characterization of a new compound from *Prunus mume* that inhibits cancer cells (*209*).

THIN LAYER RADIOCHROMATOGRAPHY

The activity of transfer ribonucleic acid (tRNA) modification enzymes was detected using radiolabeled tRNA substrates. The procedures were based on analysis of pre- or postlabeled nucleotides (with ^{32}P, ^{35}S, ^{14}C, or ^{3}H) generated after complete digestion with selected nucleases of modified tRNA isolated from cells or incubated in vitro with modifying enzyme(s). Nucleotides of the tRNA digests were separated by 2D TLC on cellulose plates to establish the base composition and identification of the nearest neighbor nucleotide of a given modified nucleotide in the tRNA sequence. Maps of 70 modified nucleotides on the TLC plates by use of two different chromatographic systems were provided (*210*).

A quantitative method for determining compounds labeled with short-lived β-emitting radionucleotides in microdialysates was based on TLC with digital photostimulated luminescence autoradiography. The lowest detectable ^{18}F radioactivity in microdyalysis fractions collected from blood of rats injected with an ^{18}F-labeled radiopharmaceutical was 0.24 Bq/application, LOQ was 0.32 Bq/application with 4−16 h exposure, and linear range was 0.1 Bq−2 kBq (*211*).

A comparison was made of radioactivity scanning, film autoradiography, and digital photostimulated luminescence (PSL) autoradiography (phosphoimaging technique) for detecting radioactivity in ^{18}F labeled synthesis products on TLC plates. It was found that radioactivity scanning is only appropriate with good TLC resolution and previously validated scanning parameters. Film autoradiography exhibited poor linearity if radioactivity varied greatly. PSL gave high sensitivity, resolution, and linearity and was rated as the best method (*212*).

Metabolism of the drug denaverine was studied by thin layer radiochromatography of urine from dosed rats. Samples were prepared by C-18 cartridge SPE and XAD-2 column cleanup, and TLC was on silica gel 60F layers with chloroform−cyclohexane−MeOH−ammonia (50:35:15:1.5) mobile phase. Radiolabeled compounds were detected and quantified by contact autoradiography, scraping and liquid scintillation counting, and radioluminography (*213*).

Other new thin layer radiochromatography applications were the ^{32}P-postlabeling-TLC assay for the in vitro detection of 8-oxodeoxyguanosine as a biomarker of oxidative DNA damage (*214*) and the determination of radiochemical purity and stability and biodistribution studies of ^{99m}Tc-kanamycin (*215*).

Joseph Sherma *received a B.S in Chemistry degree from Upsala College, East Orange, NJ, in 1955 and a Ph.D. degree in analytical chemistry from Rutgers University in 1958. He joined the faculty of Lafayette College in 1958. He is currently the John D. and Frances H. Larkin Professor Emeritus of chemistry and continues to supervise undergraduate students in analytical method development and interdisciplinary analytical chemistry−invertebrate biology research. Dr. Sherma independently and with others has written or edited over 650 papers, chapters, books, and reviews covering chromatographic and analytical methods. He was editor for residues and trace elements of the* Journal of AOAC International *for more than 20 years and is now that journal's Acquisition Editor. He is on the editorial boards of the* Journal of Planar Chromatography-Modern TLC*;* Acta Chromatographica*;* Journal of Environmental Science and Health, Part B*;* Journal of Liquid Chromatography & Related Technologies*; and* Acta Universitatis Cibiniensis, Seria F, Chemia*. Dr. Sherma has written the biennial reviews of planar chromatography published by* Analytical Chemistry *consecutively since 1970. He was recipient of the 1995 ACS Award for Research at an Undergraduate Institution sponsored by Research Corporation.*

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