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Biennial review of planar chromatography: 2011-2013

Invited Review

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Abstract: The most important advances in planar chromatography published between November 1, 2011 and November 1, 2013 are reviewed in this paper. Included are an introduction to the current status of the field; student experiments, books, and reviews; theory and fundamental studies; apparatus and techniques for sample preparation and TLC separations (sample application and plate development with the mobile phase); detection and identification of separated zones (chemical and biological detection, TLC/mass spectrometry, and TLC coupled with other spectrometric methods); techniques and instruments for quantitative analysis; preparative layer chromatography; and thin layer radiochromatography. Numerous applications to a great number of compound types and sample matrices are presented in all sections of the review.

Keywords: *Planar chromatography • Thin layer chromatography • TLC • HPTLC • Review*

1. Introduction

This is the 23rd biennial review I have written continually since 1970, all of the previous ones having been published in the American Chemical Society journal Analytical Chemistry except for the 2012 review published in the Journal of AOAC International. It covers the literature of planar chromatography found by computer-assisted searching in Chemical Abstracts (SciFinder Scholar) and the ISI Web of Science from November 1, 2011 to November 1, 2013. [Planar chromatography comprises the methods of thin layer chromatography (TLC) and high performance TLC (HPTLC), and "TLC" has been usually used in this review as a general term for methods carried out on either of these layer types without distinction.] 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), Journal of Chromatographic Science, Chromatographia, Analytical Chemistry, Journal of Liquid Chromatography & Related Technologies, Journal of AOAC International, Journal of Planar Chromatography, and Acta Chromatographica. Chemical Abstracts cited 3340 references containing the search phrase "thin layer chromatography", the main method included within the classification "planar chromatography", during the review period, compared to 3272 citations for the last review period, November 1, 2009 to November 1, 2011; the true totals are higher than these numbers because all worldwide journals publishing papers on TLC are not surveyed by this service. This publication number confirms the continuing very high significance of the field within analytical chemistry methodology. Only a small number of references reporting new research involving paper chromatography, another technique classified as planar chromatography, were considered to be important enough to be included in this review, mostly in Section 9.

Coverage is limited to the most significant papers representative of the current practice and important advances in the field of TLC, with specific sections on books and reviews, fundamental studies, methodology, equipment, and instrumentation. TLC continues to feature a broad range of applications, including analysis of pharmaceuticals, herbal medicines and dietary supplements, biological and clinical samples,

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foods and beverages, environmental pollutants, and chemicals. Especially strong trends are greater use of biological systems for detection, as in activity or effect directed analysis (EDA); hyphenation of TLC with mass spectrometry (MS); and studies of the lipophilicity of various compound types in retention studies on many different kinds of layers. Important new applications to specific analytes and sample matrices of many types are cited in virtually all sections throughout this review.

The presentations at the 17th International Symposium on Separation Sciences, News and Beauty in Separation Sciences (17th ISSS) held in Cluj-Napoca, Romania on September 5-9, 2011, and the papers contained in five special journal issues provide an up-to-date picture of many of the most important technique and application areas in TLC research over the past two years. Ten papers based on presentations at the ISSS that were published in the Journal of Planar Chromatography (2012, volume 25, number 6; guest edited by Virginia Coman) covered quantitative two dimensional (2D) TLC using a diode array detector (DAD), enantiometric resolution of chiral aromatic sulfoxides on cellulose triacetate plates, dielectroosmotic effects in dielectrochromatography, determination of some textile dyes in wastewater by solid phase extraction (SPE) followed by HPTLC, SPE and HPTLC for quantification of some antibiotics from surface waters, separation and identification of some nonsteroidal anti-inflammatory drugs using HPTLC and column high performance liquid chromatography/MS (HPLC/MS),quantum mechanics study on a series of steroids relating separation with structure, identification of anthocyanins and anthocyanidins from berry fruits to establish juice authenticity, tartrazine determination in mustard by TLC-densitometry and TLC-digital image processing, and preparation and characterization of some ethyl-phenyl modified stationary phases.

Subjects of the six papers in a Hot Topic Special Issue of the journal Medicinal Chemistry titled “TLC with Biodetection for the Screening of Natural Origin Samples” (2012, volume 8, issue 1) and guest edited by Monika Waksmundzka-Hajnos were a review of BioArena studies: unique function of endogenous formaldehyde and ozone in the antibiotic effect; separation and identification of antibacterial chamomile components using overpressured layer chromatography (OPLC), bioautography, and gas chromatography (GC)/MS; comparison of deproteinization methods used before TLC-direct bioautography and HPLC analysis of flumequine residues in milk; TLC with biodetection in the search for new drugs to treat eurodegenerative diseases; discovery of beta-glucosidase inhibitors from a chemically engineered extract prepared through ethanolysis; and 2D TLC separation of phenolic compounds from *Eupatorium cannabinium* extracts and their antioxidant activity.

A special issue of the Journal of Planar Chromaography (2013, volume 26, number 2) was published in honor of the 80th birthday of Erno Tyihak, retired scientific adviser at the Plant Protection Institute of the Hungarian Academy of Sciences, Budapest Hungary, and the inventor with Emil Mincsovics of OPLC and developer of the BioArena system for the study of the mechanism of the effect of antibiotic compounds. The papers in the special issue, guest edited by Mincsovics and Agnes Moricz, included TLC-direct bioautography for the screening of antimicrobial properties of plant extracts; detection of antibacterial activity of essential oil components by TLC-bioautography with luminescent bacteria; quantification of parabens by TLC-DAD coupled with *Vibrio fischeri* bioluminescence assay; TLC and TLC/MS screening of ursolic, oleanolic, and betulinic acids in plant extracts; TLC-densitometry of rosmarinic and caffeic acids in the evaluation of *Lamiaceae* species growing in central Europe; TLC-densitometry study of the effects of increased salinity on glucose and maltose content in *Biomphalaria glabrata* snails infected with *Schistosoma mansoni*; micro 2D TLC evaluation of the composition and antioxidative activity of selected *Mentha* sp. extracts; peculiarities of adsorption-exclusion transition of poly(methyl methacrylate) in binary mobile phases with acetonitrile (ACN) as a displacer; analysis of Hungarian wines for resveratrol by OPLC; OPLC separation of amino acids from different proteins; in situ cleanup and OPLC fractionation of chamomile flower extracts to find active components by bioautography; separation of amino acid 2,4-dinitrophenyl-5-L-valine amide diastereomeric derivatives using HPTLC and pressurized planar electrochromatography (PPEC); and planar chromatography using simultaneously flowing gas phase(s), liquid mobile phase(s), and one stationary phase (three phase chromatography). It was announced at the Balaton Symposium on HPLC, held in the resort of Siofok on Lake Balaton in Hungary in September, 2013, that the OPLC patents have been sold to a Chinese firm in Hong Kong with hope that the method might be developed into a standard herbal extract fingerprinting tool (information provided by Teresa Kowalska in a personal communication); progress in this application area of OPLC will be monitored with great interest in the future.

Subjects of papers in the yearly special issue on TLC of the Journal of Liquid Chromatography & Related Technologies [2012, volume 35, number 10), guest edited by Joseph Sherma and Bernard Fried, were hydrophilic interaction planar chromatography of geometrical

isomers of selected Co(III) complexes, chemometric characterization of model compound retention in salting out TLC on cellulose, influence of perchlorate ion concentration on the retention of fluoroquinolones on cyano (CN)-silica plates, relationship between calculated physicochemical parameters and reversed phase (RP) TLC retention behavior of alkoxy-phenylbenzamide derivatives, TLC-densitometry determination of tolperisone and its impurities 4-methylpropiophenone and piperidine in pharmaceutical preparations, TLC determination of tiapride hydrochloride and its impurities in pharmaceuticals, determination of acetylsalicyclic acid in pharmaceutical products by TLC-ultraviolet (UV) densitometry, hyphenated HPTLC for profiling some Indian natural efficiency enhancers, comparison of TLC and HPLC fingerprints of phenolic acid and flavonoid fractions derived from selected sage (Salvia) species, limits of quantification (LOQs) of some neonicotinoid insecticides, determination of pesticides in wine samples by HPLC-DAD and HPTLC-DAD, determination of food synthetic dyes in powders for jelly desserts using slit scanning densitometry and image analysis methods, and effects of crowding on neutral and polar lipid amounts and of increased salinity on glucose and maltose amounts of *Biomphalaria glabrata* snails determined by HPTLC-densitometry.

Papers in another special issue on TLC of the same journal (2013, volume 26, number 13), also guest edited by Sherma and Fried, had papers on exploration of hidden trends in classic and micellar TLC retention of model compounds by chemometric methods; support related differential impact of substituents on performance of (alkoxy-phenyl)benzamides in normal phase (NP) TLC; 2D flash test of 17alpha-ethinylestradiol and related steroids determined by fluorescence densitometry; comparison of two TLC-DPPH (2,2-diphenyl-1-picrylhydrazil) image processing procedures for studying free radical scavenging activity of compounds from selected varieties of *Medicago sativa*; simultaneous determination of carbidopa and levodopa using a new TLC method with a free radical as a detection reagent; TLC quantification of acetylsalicylic acid, caffeine, and ethoxybenzamide in combination tablets; TLC determination of glimepride and its main impurities in pharmaceuticals; TLC-densitometry determination of difloxacin and its degradation products; expansion of a model procedure for transfer of qualitative/semiquantitative TLC screening methods for fake and substandard drugs to HPTLC-densitometry methods; marker fingerprints originating from TLC and HPLC for plants of the *Lamiaceae* family; development and validation of a TLC method for analysis of synthetic foodstuffs; effects of coexposure with *Echinostoma caproni* and *Schistosoma mansoni* on neutral and polar lipids of *Biomphalaria glabrata* as determined by HPTLC-densitometry; TLC evidence of proline peptidization in solution and its enantioseparation; and optimization of variables of online injection in PPEC.

A special issue of Chromatographia (2013, volume 76, numbers 19-20) guest edited by Pawel Zarzycki included reviews on ultrathin layer chromatography (UTLC) and on planar chromatographic systems in pattern recognition and fingerprint analysis, and research papers were on a micro-TLC approach for fast screening of surface and sewage waters; studying systematic errors in the estimation decision, detection, and quantification limit for micro-TLC; a modified device for PPEC and preliminary results with online sample application; validated quantification of benzocaine in lozenges using TLC and a flatbed scanner; screening for antimicrobials in mouthwashes using HPTLC-bioluminescence detection; comparison of TLC and different micro-TLC techniques in the analysis of tropane alkaloids and their derivatives from *Datura inoxia;* miniaturized HPTLC of vitamin B12 compounds in foods; micro-2D-TLC screening of composition and antioxidative properties of plant extracts; and evaluation of free radical scavenging activity of sea urchin pigments using HPTLC with postchromatographic derivatization. Zarzycki noted in his introductory Editorial that TLC is rapidly evolving because of plate and equipment miniaturization, the discovery of new materials for stationary phases, and the availability of sensitive and selective detection methods including MS. He reported that 46% of the separation techniques for quantification, impurity tests, and substance identification protocols reported in the European Pharmacopeia are based on TLC, emphasizing its importance in pharmaceutical analysis. In light of this, Merck KGaA (EMD Millipore Corp, in the U.S.) now offers HPTLC silica gel 60 F Premium Purity plates for pharmacopeial applications (Part No. 1. 05648.0001).

A notice was received by e-mail during 2012 about the formation of the International Association for the Advancement of HPTLC (the HPTLC Association), which promotes the use HPTLC in plant analysis and other analytical fields by bringing together representatives from academia, industry, research, regulatory, and standard setting bodies. The Association's website <http://www.hptlc-association.org> describes the methodology of HPTLC, its specific purposes, benefits for members, and information on application for membership.

A bibliography service (CBS) is offered by CAMAG 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 website <http://www.camag.com> after searching for references by keywords (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 giving details of newly developed and validated TLC/HPTLC applications, *i.e.*, simultaneous determination of the pesticides temefos and fenitrothion in green tea; analysis of insulin samples from different species by HPTLC/MS; simultaneous determination of pioglitazone, metformine, and glimperide in pharmaceutical preparations; fingerprints of polysaccharides; and determination of polycyclic hydrocarbons (PAHs) in toys (issue 108; March, 2012); TLC screening for detection of Robusta admixtures in Arabica coffee, identification of polyphenolic compounds in *Rheum officinale* Baill. by TLC/MS, quantitative determination of steviol glycosides in Stevia sweetener, and identification of reaction byproducts of 4- and 5-methyl-1H-benzotriazole formed during ozonation in drinking water treatment (issue 109; September, 2012); rapid structure confirmation and quantification by HPTLC-nuclear magnetic resonance (NMR) spectrometry, bioautographic HPTLC assays for screening Gabonese medicinal plants used against Diabetes mellitus, HPTLC/MS using a novel compact single quadrupole mass spectrometer, introduction of special HPTLC and TLC plates for coupling with MS (100 um layer thickness and very low in impurities leading to better signal to noise ratio during measurements, Merck Part No. 1.00934.0001), and SPE as cleanup for pesticide residue analysis of tea samples using planar chromatographic techniques (issue 110; March, 2013); and quantification of alkaloids in *Sceletium tortuosum;* determination of caffeine, taurine, and arginine in shampoos; screening method to study the reactivity of cosmetic UV filters on skin proteins; identification of acetylcholinesterase (ACE) inhibitors from Galbanum; and TLC fingerprint of German propolis (issue 111; September, 2013). CAMAG also publishes a newsletter on new instruments and methods titled “CAMAGflash” that is distrubuted electronically on an irregular schedule; those interested can subscribe on the CAMAG website.

Professor Luciano Lepri celebrated his 70th birthday on January 2, 2011, and a letter describing his research work, especially the chiral TLC resolution of enantiomers belonging to important classes of organic compounds, was published in the Journal of Planar Chromatography [1]. Editorials in Acta Chromatographica [2] and the Journal of Planar Chromatography [3] were written in honor of the 80th birthday of Friedrich (Fritz) Geiss in 2012; his most important contribution to TLC was probably the book “Fundamentals of Thin Layer Chromatography” published in English in 1987 by Huethig (originally published in 1972 in German), which clearly and comprehensively explained the complex separation mechanisms occurring in TLC systems. It is with sadness that I report the death of the noted TLC researcher Henryk Lamparczyk on November 16, 2012; his outstanding accomplishments were described by Zarzycki in the Editorial introducing the special issue of Chromatographia mentioned above.

2. Student experiments, books, and reviews

Student experiments for use in teaching TLC were described for high school laboratories to study the synthesis of adenine in a model prebiotic reaction [4], and for college organic chemistry laboratory courses to identify unknown compounds in essential oils [5] and to characterize a mixture of capsaicinoids [6].

Two books on fingerprint analysis of herbal drugs [7,8]; a general book on the fundamentals, methods, and applications of HPTLC [9]; and a three-book series by P.D. Sethi on HPTLC quantitative analysis of pharmaceutical formulations that was published by CBS Publishers & Distributors Pvt. Ltd., New Delhi, India, and reviewed by the Editor in Chief of the Journal of Planar Chromatography, Bernd Spangenberg [10], were published. An older book describing the basis of TLC by the famous chromatography expert Raymond P.W. Scott with the latest reference from the year 2000 is fully available online free of charge at <http://www.chromatography-online.org/TLC/contents.html>. A book titled Thin Layer Chromatography in Drug Analysis, coedited by Lukasz Komsta, Waksmundzka-Hajnos, and Sherma, will be published in 2014 with 1067 pages on techniques and applications for all types of drug compounds by CRC Press/Taylor & Francis Group as Volume 106 of their Chromatographic Science Series edited by Nelu Grinberg.

Comprehensive review of TLC applications to specific compound classes is not possible in this article because of space restrictions. However, information on TLC applications is available in the following review articles: determination of fluoroquinolones in biological matrices and pharmaceutical formulations [11,12]; method development and validation steps for HPTLC assays of active ingredients in pharmaceutical formulations [13]; developments in mycotoxin analysis from 2010 to 2012 [14]; determination of selective serotonin reuptake inhibitor antidepressants [15]; analysis of alkaloids from different chemical groups [16]; amino acid and vitamin determinations [17]; chiral derivatizations applied to the separation of unusual amino acid enantiomers [18];

quality control (QC) of pharmaceuticals [19]; extraction and TLC determination of phospholipids in biological samples [20]; methods for extraction, preconcentration, and determination of quercetin and other flavonoids [21]; analysis of amino acids [22]; and techniques for determination of aflatoxins in different foodstuffs [23]. Advances in the TLC of pesticides is reviewed biennially by Sherma in odd numbered years [24]. A scientometric study of the development of TLC in the 1980-1990 and 2000-2010 decades based on the analysis of the content of published papers was presented [25].

3. Theory and fundamental studies

This section refers to a selection of studies of TLC retention and lipophilicity (hydrophobicity) characteristics for a variety of compounds that were chosen as examples of active research areas. These references also illustrate many of the different types of layers and mobile phases used for TLC at this time. Almost all of the layers used contained an indicator (phosphor) that fluoresces bright green or blue when excited with 254 nm UV light (called an F layer), and compounds that absorb this light are detected as black zones or spots that quench the fluorescent background on the F layer. (The terms “zone”, “spot”, and “band” are generally used interchangeably in the TLC literature and will be in this review, although in exact usage “zone” is a general term while “band” and “spot” indicate different zone shapes.)

QSRR (quantitative structure-chromatographic retention relationship) studies as a useful tool to characterize chromatographic systems and their potential to simulate biological processes were reviewed from a medicinal chemistry point of view [26]. A QSSR was built by means of multiple linear regression (MLR) and partial least squares (PLS) regression to investigate the relationship between the structural descriptors of 23 newly synthesized N-substituted 2-alkylidene-4-oxothiazolidines on octadecylsilane (termed C18 or RP18) and CN bonded layers using methanol (MeOH), ACN, and tetrahydrofuran (THF) as mobile phase organic modifiers [27]. A structure-activity relationship study of selected compounds with activity on dopaminergic, serotoninergic, and muscarinic receptors was carried out on NP plates impregnated with a solution of L-aspartic acid using the statistical tool stepwise discriminant analysis (SDA) [28].

Other published retention studies included correlation between retention data and molecular descriptors of antiepileptic hydantoin analogs using RP-TLC and stepwise and PLS regression [29]; QSRR analysis of some xylofuranose derivatives by the linear multivariate method and NP-TLC [30]; QSRR study of the TLC behavior of aldopentose derivatives on silica gel impregnated with paraffin oil (RP mechanism) and binary mobile phases of MeOH, acetone (ACT), and dioxane (DIOX) with water [31]; behavior of ivabradine using aqueous and nonaqueous mobile phases with silica gel 60 Fs (s designates an acid stable fluorescent indicator), C2 (RP2, ethyl bonded) Fs, C8 (RP8, octylsilyl bonded) Fs, C18 Fs, C18 W Fs (W designates water wettable), CN Fs, diol (two OH groups) Fs, amino (NH_2) bonded Fs, aluminum oxide 150 F, cellulose F, and polyamide F layers [32]; influence of temperature on retention of bile acids in silica gel TLC and the role of the steroid skeleton [33]; effect of mobile phase composition on the elution of phenylacetone on alumina layers with 1,4-DIOX mobile phase [34]; quantitative relationships between molecular descriptors, retention behavior, and in vitro antituberculosis activity of phytol derivatives on C18 and CN plates with MeOH- and ACN-water binary mobile phases [35]; and QSRR study of 31 newly synthesized polyoxygenated steroids on C18 layers [36].

Lipophilicity of a compound is a physicochemical property of crucial importance in medicinal chemistry because it defines pharmacokinetics and pharmacodynamics of a drug substance. Methods of determining lipophilicity and its role in medicinal chemistry [37] and the influence of the structure of bile acids on their lipophilicity as expressed via the retention parameters in RP-TLC [38] were reviewed. The following are representative examples of the many papers published on lipophilicity determination using different TLC plates and chemometric approaches: pyrrolyl-acetic acid derivative inhibitors of aldose reductase on C18 and C8 plates [39]; comparison of the predictive ability of RP-TLC and RP-HPLC with C18 W F plates and MeOH-water mobile phases for eight new *p*-toluenesulfonyl-hydrazinothiazole and hydrazine-bis-thiazole derivatives [40]; demonstration that CN and C18 layers are superior to silica gel as a lipophilicity determination tool carried out by development with various mobile phases in a Chromdes horizontal DS Teflon chamber [41]; natural toxins with MeOH-water mobile phases on C18, C18 W, C8, C2, NH_2, CN, and diol plates [42]; ACE inhibitors and hydrochlorothiazide on cellulose in the RP mode with three binary solvent systems [43]; biologically active analogs of flavonoids on silica gel plates impregnated with silicone oil and acetone-citrate buffer pH 3 (1:1) mobile phase [44]; and 32 novel ACE inhibitors-1,2,3.4-tetrahydroacridine and 2,3-dihydro-1H-cyclopenta[b]quinoline derivatives-with C18, C8, C2, CN, and NH_2 plates and DIOX-citrate buffer pH 3 binary mobile phases [45].

The mobile phase motion in ascending micellar TLC with NP plates was investigated, and the applicability of a quadratic relationship between the time of mobile phase motion and the distance from the mobile phase entry position and its front was validated; R_f values of hydrophilic test compounds changed significantly only at low contents of modifier in the micellar mobile phase [46]. Lateral movement of chiral analytes was studied on plain silica gel layers and those impregnated with L- and DL-arginine; the observed lateral relocation of the 2-phenylpropionic acid enantiomers was interpreted as a nonlinear motion resembling that of propeller-like chiral rotors [47]. Localization, or noncovalent attachment of molecules of solute or solvent to strong sites that form part of the adsorbent surface, was studied in terms of its significance in adsorption TLC and various effects on sample retention and resolution [48].

Chemometric methods are widely used in studies such as those reported in this section, and it is expected that they will be used much more in the future in TLC for these as well as for optimization of various steps such as sample preparation and mobile phase composition as well as analytical data handling.

4. Chromatographic systems

4.1. Stationary phases

In TLC the prepared test sample is applied to the chosen stationary phase, and the plate is developed with the mobile phase to allow the separation to occur. Then the plate is dried, and various methods can be applied to obtain detection, qualitative analysis, and quantification of the compound zones. These steps in the process of TLC are covered in the following sections.

Section 3 above contains references on retention and lipophilicity studies in which virtually all of the great variety of commercially available chemically bonded silica gel and nonbonded plates were used, in addition to impregnated plates to make silica gel function with an RP separation mechanism. Use of Merck silica gel 60 F plates containing silica gel with a 60 angstrom (6 nm) pore diameter and phosphor fluorescing at 254 nm (designated F_{254} plates) is by far most widely reported in the literature; the subscript 254 with the F will not be used in this review, and the presence of the phosphor will not always be specified when the plate type is given although it is usually present because it allows detection of many compounds by fluorescence quenching and does not usually interfere with separations or other detection procedures. HPTLC plates with smaller particle size sorbents and thinner layers offer faster, more efficient separations with better resolution than TLC plates. Other companies manufacturing plates that are specified in literature references are Analtech Inc. and Macherey Nagel. Sale of Whatman plates was discontinued by GE Healthcare as of mid-April, 2012, leaving a void in availability from any manufacturer of some unique plates Whatman offered such as channeled C18 plates with a preadsorbent zone and Multi-K plates with a strip of C18 sorbent adjacent to an analytical layer of silica gel, and vice versa, on which 2D TLC with NP and RP mechanisms in the two directions could be carried out.

This section will selectively review papers reporting analyses mostly on layers other than plain silica gel. Additional diverse stationary phase/mobile phase systems are described in other sections of this review.

All currently used stationary phases for modern TLC and HPTLC [49], chiral stationary phases for enantioseparations [50], and amino acids as chiral selectors in layer impregnation and as mobile phase additives [51] were reviewed.

Most commercial TLC plates are prepared from irregular sorbent particles; plates with spherical silica gel particles (LiChrospher) were used for densitometric determination of rosmarinic acid in different matrices with toluene (TOL)-ethyl formate-formic acid (HCOOH) (6:4:1) mobile phase [52] and separation of polystyrene using cyclohexane (CY)-THF (78:22) mobile phase [53]. Silica gel 60 G F HPTLC plates with gypsum binder instead of the usual organic binder were applied for the densitometric quantification of oleanolic acid from the roots of *Helicteres isora* Linn. [54]. Laboratory prepared layers of Ti(IV) silicate ion exchange layers with KBr-MeOH (9:1) mobile phase were used for densitometric determination of streptomycin in dosage forms and biological fluids [55] and layers of cellulose tribenzoate for enantiomeric resolution and densitometry of chiral aromatic sulfoxides with detection by exposure to iodine vapor [56]. The design, preparation, and characterization of a chiral molecular imprinted polymer (MIP) layer was reported for determination of the enantiomeric purity of naproxen [57]. 2D TLC with a stationary phase gradient made by connecting silica gel and cellulose layers allowed high resolution separations of hydrophilic vitamins [58].

Layers can be impregnated or modified with various chemicals to improve resolution of certain mixtures. Examples include enantioresolution of some beta-blockers and a beta2-agonist using silica gel impregnated with the ligand exchange reagents Cu(II)-L-threonine, -tartaric acid, or -serine [59]; alumina and talc-alumina layers impregnated with triaryl phosphate for the separation of four amino acids by development with dimethyl sulfoxide (DMSO)-1 M HCl (1:1) [60]; silica

gel H plates (H indicates no foreign binder) impregnated with microcrystalline cellulose gel for the TLC of heavy metal cations with 10% aqueous KSCN-Triton X-100 (1:1) mobile phase [61]; silica gel plate impregnated with 6% caffeine for quantification of PAHs in environmental samples using image analysis with a digital camera and personal computer [62]; silver ion impregnated silica gel to fractionate the fatty acids in milk and feed based on degree of unsaturation [63]; and TLC separation of wheatgrass pigments on sucrose impregnated silica gel plates to decrease pigment degradation [64].

There was a significant surge in papers reporting preparation of nanostructure, ultrathin, and other new types of layer stationary phases, and it is expected that research activity in this area will continue increase in the fture. The following are selected examples of reported new layers. Penicillins, cephalosporins, and carbapenems were separated on transition metal silicate modified silica layers with detection by iodine vapor [65]. Losartan potassium and hydrochlorothiazide in pure form and tablets were simultaneously determined using a C18 modified Aleppo bentonite layer with pH 3.2 ammonium phosphate buffer-ACN (6:4) mobile phase [66]. Florescence quenching detection of explosives was improved by covalently bonding the fluorescent organosilicon copolymers poly(silafluorenyldiethylnylspirobifluorene) and poly(tetrasilolediethynylspirobifluorene) to silica gel support through the use of a trimethoxysilyl end group [67]. High resolution drug separations were carried out by use of gradient stationary phases formed on activated HPTLC plates using “controlled rate infusion” in which SiOH groups were reacted with 3-aminopropyltriethoxysilane in a time dependent fashion using infusion with a programmable syringe pump [68]. Silver impregnated silica gel separations of fatty acid methyl esters were improved by use of new silver thiolate layers that were more light stable [69].

Nanostructures for UTLC were prepared by combining glancing angle deposition (GLAD) and atomic layer deposition (ALD), and their use was investigated for lipophilic dye separations [70]. Electrospun polyvinyl alcohol (PVA) UTLC plates were fabricated using an in situ crosslinking technique and characterized for the separation of amino acids and fluorescein isothiocyanate labeled amino acids followed by detection with ninhydrin reagent [71]. Polarity adjustable RP-UTLC plates were fabricated on glass substrates with SiO_2 nanopillars deposited using the GLAD technique and functionalized with C18 groups [72]. A mixture of oxidized multiwalled carbon nanotubes (MWCNTs) and D-(-)-tartaric acid impregnated silica gel was used as the stationary phase for TLC enantioseparation of ofloxacin enantiomers [73]. Effects of catalyst thickness on the fabrication and performance of carbon nanotube templated TLC plates was studied, and the best separation of caffeine, phenacetin, and propyphenazone was obtained on an NP plate grown with 5 nm of Fe catalyst [74]. Ultrathin electrospun nanofiber layers with incorporated photoluminescence indicator (Mn activated Zn silicate) were fabricated to transfer and enlarge the detection applications of UV active compounds; the separation of seven preservatives and a beverage sample were studied, and the layers were found suited for integration into the Office Chromatography concept (Section 5.2) and were hyphenated with the CAMAG elution-head based TLC-MS interface (Section 6.3) [75]. Ozonation was reported of patterned, vertically aligned CNT forests as a method of priming them for pseudo-ALD of silica to produce microfabricated CNT templated TLC plates [76]. The following instrumental methods were applied to characterize the surfaces of materials in microfabricated CNT templated TLC plates: x-ray photoelectron spectrometry (XPS), valence band spectrometry, positive and negative ion time of flight secondary ion MS (TOF-SIMS), Rutherford backscattering spectrometry (RBS), and He ion spectrometry [77]. MWCNT impregnated TLC plates were prepared by spreading a slurry of silica gel G F, D-tartaric acid, MWCNTs, and sodium carboxymethylcellulose solution, and they were used for the enantioseparation of ofloxacin with ACN-BuOH (1:1) mobile phase; a general discussion on the potential of MWCNTs in chromatographic enantioseparations was presented [78]. An improved microfabrication process was described for preparation of TLC plates from patterned CNT forests via direct infiltration/coating of the CNTs by low pressure chemical vapor deposition of Si from SiH_4 followed by high temperature oxidation of the CNTs and Si [79]. Aligned electrospun polyacrylonitrile nanofibers for UTLC were fabricated and characterized by separation of a mixture of beta-blockers and steroids; compared to nonaligned electrospun UTLC plates, the aligned plates were 2-2.5 times faster and had twice as much reproducibility and 100 times greater efficiency [80]. D-Tartaric acid modified MWCNTs were prepared and used as chiral impregnating reagents for TLC enantioseparation of propranolol enantiomers with ACN-tert. butanol (BuOH)-acetic acid (HAc) (49:49:2) mobile phase [81].

Merck has discontinued manufacture of its UTLC monolithic silica gel plates. Characteristics and practical applications of the new plates described above may not be studied in depth unless they become commercially available.

4.2. Mobile phases

Mobile phases are usually chosen after trial and error testing of solvent mixtures with appropriate strengths (R_f values of 0.2-0.8 are usually optimum) and selectivities relative to the layer and mixture to be separated, guided by literature searching and personal experience in designing TLC systems. The use of various systematic optimization systems that were previously developed, such as PRISMA, is seldom reported in TLC journal articles, and, in fact, it is only rarely explained how the mobile phase was selected.

However, three new papers were published related to mobile phase optimization. A method termed "design of experiment" was described to choose a mobile phase for HPTLC analysis of gatifloxacin and related substances; preliminary screening of 22 solvents was first performed by uniform design to establish the mobile phase to be optimized, then the optimal proportions of components were chosen by central composite design [82]. A new RP-HPTLC method was developed for separation of isoniazid, ethambutol, rifampicin, and pyrazinamide in fixed combination antituberculosis tablets using systematic optimization of the composition of alcohol-water mobile phases using the window diagramming concept to obtain the best separation [83]. A new method of solvent classification was proposed based on the five system constants of the solvent parameter model for transfer of neutral compounds from the gas phase to solvent and hierarchical cluster analysis for identifying solvents with similar properties and organizing them into groups; the method resulted in classification of 36 common solvents used in TLC into seven selectivity groups, with four solvents behaving independently [84].

The following are examples of mobile phase additives used to improve TLC analyses. Escitalopram oxalate was enantiomerically assayed in the presence of in process impurities using beta-cyclodextrin (CD) and urea as two different chiral selectors in mobile phases for silica gel 60 F plates [85]. beta-CD was also used as a mobile phase additive in the study of (+)-(S)-ibuprofen enantiomer chiral inversion on RP18 F plates with densitometry [86]. MeOH-ACT (1:1) mobile phase with 0.1% of 2-tert-butylhydroquinone added to increase pigment stability was used in the HPTLC-densitometry screening of major dietary carotenoids and quantification of lutein in food supplements on C18 layers [87]. A TLC system comprising silica gel stationary phase and 1% aqueous urea solution (pH 7.44) gave good resolution of amino acid mixtures present in pharmaceutical formulations during analysis by densitometry [88]. ACN-water (6:4) mobile phase with addition of 1.5% of an imidazolium class ionic liquid was used to obtain the best separation of the basic analyte naphazoline in the HPTLC-densitometry assay of a pharmaceutical formulation on RP18 Fs layers [89]. The TLC of poly(methyl methacrylate) was carried out using methyl ethyl ketone (MEK) as a strong solvent displacer [90]. Identification and separation of Pb(II), Ni(II), and Co(II) in water samples on silica gel 60 F HPTLC plates was achieved by using 0.2 M aqueous sodium dodecyl sulfate (SDS) surfactant-0.08 M oxalic acid (1:9) mobile phase [91].

Additional stationary and mobile phase combinations are specified for TLC/HPTLC separations reported in other sections of this review. A future prospect is that there will be emphasis on the use of only "green" environmentally friendly solvents in mobile phases such as methanol, ethanol, ethyl acetate, acetone, and toluene, and elimination of the use of chlorinated solvents, benzene, and acetonitrile among others.

5. Apparatus and techniques

5.1. Sample preparation

In TLC, the complete sample is contained in the chromatogram between the origin and the mobile phase front at the top of the plate. Because the plate is not reused strongly retained components at or just above the origin are no problem, whereas in an HPLC run the strongly retained sample components may be irreversibly sorbed on the column and change its performance for later runs, or they may elute later and interfere with subsequently injected samples. Therefore, sample purification (cleanup) is more critical in HPLC compared to TLC. The usual sample preparation for TLC still involves classical techniques such as simple dissolving; protein precipitation; lyophilization; traditional extraction in a flask, separatory funnel, or Soxhlet apparatus or by refluxing; and cleanup by liquid-liquid partitioning and/or column chromatography (usually on silica gel) if required. Some liquid samples, such as beverages, can be applied to the layer directly with no sample preparation. Tablet pharmaceutical formulations are usually ground, and the active ingredient is dissolved by sonication and/or magnetic stirring and applied to the plate after 0.45 um syringe filtration removal of any undissolved inactive ingredients. This section gives examples of sample preparation procedures used prior to TLC determination. Additional sample preparation methods, such as liquid-liquid microextraction (LLME), are mentioned in many of the applications described below.

A method for quantification of nifedipine in human serum included liquid-liquid extraction (LLE) and use

of carbamazepine as an internal standard; internal standards are mostly used in TLC-densitometry of clinical samples while external standarization is used otherwise [92]. SPE on C18 cartridges and Ni(II) impregnated silica gel plates with 2-propanol (PrOH)-dichloromethane (DCM) (7:3) mobile phase were applied for the densitometric determination of the antidepressant drugs fluvoxamine maleate, paroxetine, and sertraline in human plasma [93]. Ethyl acatate (EtAc) extraction followed by two column enrichments on XAD resin and Sephadex LH-20 size exclusion chromatography (SEC) sorbent were used in the silica gel HPTLC-densitometry analysis of *Eugenia jambolana* Willd. ex O. Berg for its major anthocyanins [94]. Solvent extraction, solvent partitioning, and cleanup on Sephadex and polyamide columns were required prior to the determination of rutin, narcissin, nicotiflorin, and isoquercitrin in *Caragana spinosa* shoots using HPTLC silica gel plates with double development by EtAc-1,2-dichloroethane-HAc-85% HCOOH-water (10:2.5:1:1:0.8) [95]. Supercritical fluid extraction (SFE) and ultrasound assisted extraction (UAE) were found to recover the essential oils, flavonoids, and antioxidants from *Anacardium occidentale* leaves prior to TLC analysis [96]. Flavonoids were extracted from foods of plant origin by means of accelerated solvent extraction (ASE) with ACT-water (7:3) in a study of the dietary intake of specific flavonoids using TLC and densitometry with a CAMAG Scanner 3 [97]. Fish oil samples were applied to silica gel 60 F HPTLC plates after saponification and alumina column cleanup in the determination of vitamin D3 by scanning at 280 nm after development with chloroform (CHL)-diethyl ether (DEE)(9:1) mobile phase [98]. A comparison of Soxhlet extraction, UAE, and maceration extraction showed that UAE was best for recovery of the polyphenols from *Bauhania purpurea* leaves prior to determination of antioxidant and antimicrobial activity by TLC-bioautography [99]. In a comparison of percolation extraction, UAE, and microwave assisted extraction (MAE), UAE with aqueous HAc or tartaric acid was found to be best for recovery of atropine and scopolamine alkaloids from *Datura innoxia* Mill., and LLE using DCM was found to be better than SPE with a cation exchanger or C18 sorbent for purification of the extracts prior to silica gel 60 F HPTLC in a horizontal DS chamber with MeOH-ACT-aqueous ammonia (5:4.5:0.5) mobile phase [100]. ASE was superior to conventional soaking in MeOH for quantification of bergenin from *Mallotus philippinensis* on silica gel 60 F using EtAc-MeOH-HAc-HCOOH (8:1:0.5:0.5) mobile phase and scanning at 284 nm; confirmation of identity was obtained by extraction of zones with MeOH and online transfer through an interface into an electrospray ionization (ESI) ion trap (IT) mass spectrometer [101]. A method based on LLME combined with ion exchange HPTLC on stannic silicate layers developed with TOL-EtAc (10:1) mobile phase and scanning at 280 nm was applied for determination of plasticizers in aqueous samples [102].

5.2. TLC

Although modern TLC is not a fully automated method as is HPLC, various steps of TLC are automated by use of instruments with the advantage that each step can be optimized for analysis of individual sample components. The automated instruments most used throughout the world for TLC-densitometry analyses include the CAMAG Linomat 4 or 5 for sample/standard solution application, the CAMAG ADC 2 for layer development with the mobile phase, the CAMAG Scanner 3 or 4 for detection and quantification of zones, and the CAMAG TLC-MS interface for confirmation of the identity of zones. These four instruments were used for the determination of *p*-coumaric acid, *trans*-resveratrol, and pterostilbene in bacterial cultures, food supplements, and wine with detection by three postchromatographic derivatization reagents [anisaldehyde, sulfuric acid-ethanol, and phosphomolybdic acid (PMA)] and documentation of chromatograms with a CAMAG Digi-Store 2 [103].

Initial zones are applied manually with a micropipet in most TLC work, but instrumental standard and sample bandwise application is recommended for highest accuracy and precision in quantitative analysis by densitometry. To obtain separations and quantitative results as close as possible to those resulting from instrumental application, manual application with a micropipet should be carried out on a plate with a preadsorbent area that automatically forms and focuses band shaped zones during development. Numerous successful quantitative HPTLC-densitometry analyses have been reported in which manual application with a Drummond digital microdispenser onto preadsorbent plates was used, *e.g.* a study of the effects of *Schistosoma mansoni* on the neutral and polar lipid amounts in various mouse organs [104].

The vast majority of TLC analyses are carried out by capillary flow one dimensional (1D) ascending development in a mobile phase vapor saturated large volume chamber (N-chamber), a CAMAG twin trough chamber (TTC), or a CAMAG ADC 2 at ambient laboratory temperature. The TTC is an N-chamber modified with an inverted V-shaped ridge in the bottom dividing the tank into two sections that allow development with a small volume of mobile phase as well as equilibration with the vapors of the mobile phase or other solutions if desirable for increased selectivity. Automatic ascending

development in the ADC 2 with controlled conditions of mobile phase distance, chamber saturation and humidity, and final drying can be used when methods must comply with GMP (Good Manufacturing Practice)/GLP (Good Laboratory Practice), IQ (Instrument Qualification)/OQ (Operation Qualification), and 21 U.S. Food and Drug Administration (FDA) Code of Federal Regulations (CFR) Part 11 requirements. A saturated, preconditioned ADC2 chamber was used in a validated silica gel 60 F HPTLC-densitometry method for quantification of beta-D-glucoside and four major phenolic compounds in vanilla fruits, beans, and extracts with *n*-hexane (HEX)-CHL-MeOH-HAc (5:36:4:0.5) mobile phase [105].

Temperature controlled micro-TLC on RP18 W and RP18 W Fs plates in a homemade temperature controlled horizontal chamber unit was applied for separation and preliminary screening of steroid fractions from biological and environmental samples [106]. Low temperature TLC performed using a horizontal DS chamber [41,100] in a refrigerator at -10°C were demonstrated for detection of free radical scavengers and ACE inhibitors in volatile samples on silica gel and CN layers with the DPPH test; samples were applied to plates as spots using a Desaga DS-30 automatic TLC sampler [107].

The offline combination of TLC, HPLC-DAD, and quadrupole (Q)-time of flight (TOF) ESI MS resulted in successful separation and identification of spinochrome pigments from sea urchin. Two fractions of pigments were separated by TLC on oxalic acid treated silica gel 60 F plates by development in a TTC with CHL-MeOH-HAc-water (50:11:5:2) and eluted with MeOH using a CAMAG TLC-MS interface [108].

The influence of a static homogeneous magnetic field generated by a pair of permanent magnets on TLC retention and efficiency results was explained [109]. A CAMAG horizontal developing chamber under sandwich configuration was used for silica gel 60 F TLC with EtAc-MeOH-ammonia 2.8% (20:10:4) mobile phase in the validated HPTLC-densitometry (263 nm) determination of nicotine in tobacco extracts [110]. A new concept of coupling sample preparation by TLC with effect directed analysis (EDA) was proposed for screening water for estrogenic activity using planar-YES (a variation of the yeast estrogen screen) as an example [111]. An atomic force microscope (AFM) was presented as an instrument for rapid, miniaturized chromatography; the AFM was used to inject a sample, provide shear driven liquid flow over a functionalized substrate with NP chemistry, and detect separated components that were then analyzed with surface enhanced Raman spectrometry (SERS) using AFM deposition of gold nanoparticles on the separated bands of lipophilic dyes tested as model compounds [112]. An ion pair TLC method was reported for separation and purification of imidazolium based ionic compound mixtures using aluminum foil backed silica gel plates (usually called silica gel aluminum plates as opposed to silica gel glass plates with glass backing) dipped into 6% (w/v) KBr solution for 15 min, air dried for 15 min, and developed with 10-30% MeOH-DCM mobile phases; compounds were detected with Dragendorff-Munier bismuth nitrate reagent as orange zones [113]. In hydrophilic interaction chromatography (HILIC), highly polar substances can be separated on a neutral hydrophilic stationary phase using a hydrophobic or mostly organic mobile phase with a mechanism of liquid-liquid partition; this method was used on HPTLC silica gel plates with deoxycholate-ACN (1:5) mobile phase for separation and identification of pentose, hexose, and disaccharides in cough syrup, multivitamin syrup, and human blood [114]. Bioassay guided isolation and identification of antimicrobial compounds from thyme essential oil were made using forced flow OPLC with online detection and fractionation, bioautography, and GC/MS [115]. The separation mechanism of eight Co(III) complexes with ethylenediaminetetraacetic acid (EDTA)-type ligands during salting out TLC was studied; silica gel impregnated with polyethylene glycols and developed with ammonium sulfate solutions as mobile phases as well as unimpregnated silica gel developed with different salt solutions were evaluated [116]. A novel "target constituent knock out" strategy coupling TLC, ultra-performance column LC with an evaporative light scattering detector (UPLC-ELSD), and microcalorimetry was applied to the preliminary screening of antibacterial constituents from *Calculus bovis*; six single constituents in the bovis samples were "knocked out" on silica gel TLC plates, they were identified by UPLC-ELSD, antibacterial activity on *Staphylococcus aureus* was evaluated by microcalorimetry combined with principal component analysis (PCA), and the interaction properties between these single constituents were elucidated [117].

A miniaturized UTLC system that was developed by Gerda Morlock and coworkers and discussed in my last biennial review has become known as "office TLC" [75]. With the goal of faster analyses, reduced solvent and sample volumes, and lower costs, the office TLC framework with GLAD UTLC plates was demonstrated for the qualitative analysis of sugars in a commercially available chocolate sample. A consumer inkjet printer was used to apply very sharp 3-30 nL bands of lactose, sucrose, and fructose, and successful coupling to ESI-MS was obtained. Limit of detection (LOD) values for the sugars was experimentally proven down to 60-70 ng per band [118]. One of the greatest advantages of TLC compared to HPLC is the ability to chromatograph multiple samples and standards side by side on a single

plate under essentially identical conditions. Morlock and Frank Gamlich illustrated the high throughput conventional silica gel HPTLC separation of up to 21 samples in 20 min in the fingerprint analysis of plant polysaccharides used as thickening agents with selective detection using aniline diphenylamine *o*-phosphoric acid postchromatographic derivatization reagent [119].

Multiple development on a single plate with drying between runs can be used to increase resolution of mixtures. In addition to double development with the same mobile phase mentioned above [95], two different mobile phases can be used, *e.g.* ACN-water-MeOH-HCOOH (20:20:1:0.005 and 20:17:1:0.005) for the dual 80 mm development of C18 F plates in the simultaneous quantification of flavonoids and bioflavonoids in *Ginkgo biloba* [120]. Silica gel 60 F plates were developed with HEX-EtAc (7:3) followed by DCM-DEE (8:2) for screening various *Juniperus* species for occurrence of umbelliferone with determination by fluorescence densitometry [121]. Three mobile phases were used for development of silica gel 60 F plates for different distances in the quantification of arbutin and hydroquinone in herbal drugs: MeOH for 1 cm, then EtAc-MeOH-water (77:13:10) for 5.5 cm for separation of arbutin, and finally *n*-heptane (HEP)-EtAc (1:1) for 4.5 cm to separate hydroquinone; at each step, the ballast material was cut away [122]. Another three step bivariant multiple development sequence was used for quantitative analysis of herbal drugs on 10×10 cm silica gel 60 F plates in a Chromdes horizontal DS chamber: alkaloids were separated at a distance of 6 cm into two zones using TOL-EtAc-MeOH (1:1:1), the plate was cut at 4.5 cm into lower (containing berberine and chelerithrine) and upper (with chelidonine and other compounds) parts, and the upper part was developed with *n*-PrOH-HCOOH-water (84:8:8) for 4.5 cm and the lower with EtAc-HCOOH-water (7:1:1) for 5 cm to complete the separations [123]. Plant saponins and sugars from germplasm collections of the subgenus Soja were resolved by development of a silica gel plate with CHL-MeOH-water (65:35:10) and then 10% sulfuric acid; the sulfuric acid dehydrated the sugars and reduced their interactions with the layer, shifting the sugars above the saponins on the plate during the second run [124]. Simultaneous quantification of water- and oil-soluble sunscreens was done by development of a silica gel 60 F plate with CY-DEE (5:1); densitometry at 360 and 300 nm for separated avobenzone and octyl salicylate, respectively; development with EtAc-ethanol (EtOH)-water (75:35:30); and finally densitometry at 300 nm for phenylbenzidazol sulfonic acid [125]. Automated multiple development with the CAMAG AMD instrument on caffeine impregnated silica gel HPTLC plates separated heavy petroleum products according to their number of aromatic rings; the elution gradient comprised DCM-HEP (70:30) to (65:35) in 34 elution steps at 2 mm per step over a distance of 68 mm [126].

Other methods to increase separation power of TLC are 2D development, in which a sample is applied in a corner of a plate that is developed using two mobile phases with complementary selectively in perpendicular directions; multidimensional separations using TLC combined with a different method; and gradient elution TLC without AMD.

The 2D TLC determination of phenolic antioxidants from *Eupatorium cannabinum* extracts was carried on CN bonded layers with HEP-2-PrOH and HEP-EtAc mobile phases in the first direction (NP mechanism) and MeOH-water mobile phases in the second direction (RP mechanism); detection spray reagents used were 2-(diphenylboryoxy)-ethylamine and polyethylene glycol (PEG) 4000 or DPPH [127]. A novel apparatus with a dual solvent reservoir designed to apply a concurrent orthogonal electric field during TLC was described and characterized using separations of various vitamins, amino acids, and dyes; improved separation with equivalent analysis time was reported compared to TLC alone [128]. A new horizontal developing chamber was illustrated for the separation of a 10 dye mixture on RP18 W plates using four step linear, convex, and concave MeOH-water mobile phase gradients; practical advantages were shown in comparison to other chambers [129].

The research group of Victor Berezkin in Russia continued their studies of TLC apparatus and techniques with the following papers: a review of the history of development of TLC sandwich (S)-chambers of different types with small and zero gas volume to promote future use [130]; a new N-chamber containing a cassette holder with a TLC plate previously wetted by the mobile phase (a “feedng plate”) that is placed 0.2-1 mm from the analytical plate to accelerate mobile phase vapor saturation [131]; an S-chamber with a dry counter plate positioned a small distance above the analytical plate with both layers turned to each other gave up to 50% increase in R_f value, 2-2.5 times more separation efficiency, and 25% increase in zone resolution [132]; and comparison of the popular DS horizontal chamber with an “S-min” chamber with minimum gas volume [133].

PPEC, in which the mobile phase is driven by electroosmotic flow while the system is pressurized in a manner that allows heat flow between the sorbent layer and pressurizing medium, was developed around 2004 by the research group of David Nurok and continues to be researched by Tadeusz Dzido and coworkers.

An example of recent work is the simultaneous determination of acetaminophen, propyphenazone, and caffeine in cefalgin preparation. A C18 layer, ACN-buffer mobile phase, and 1.8 kV polarization voltage gave a separation time of 6 min compared to 20 min and better separation than for HPTLC alone with the same layer and mobile phase [134].

Iatroscan TLC on thin layer quartz rods (Chromarods) with a flame ionization detector (FID) is mainly applied to the determination of hydrocarbons and lipids that do not have a UV active chromophore in their structures. Two applications published in the review period are calibration and accurate weight determination of clean saturates, aromatics, resins, and asphaltenes (SARA) in Athabasca heavy crude oil [135], and the extraction of lipids from abalone by CO_2 SFE and enzyme assisted organic solvent extraction followed by determination by TLC-FID [136].

6. Detection and identification of TLC zones

Zone detection (also termed visualization) in TLC is based on natural color, fluorescence, or UV absorption (fluorescence quenching on an F layer) or on the use of various universal or selective chemical or biological detection reagents applied by spraying or dipping or through the vapor phase (*e.g.* iodine [56,65]). 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 a chromatogram to increase the amount of information obtained; analytes do not necessarily have to be separated in order to obtain their successive quantification by use of different detection methods. 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 (Sections 6.3 and 6.4). Chromatogram images can be documented with a photographic system like the CAMAG Digi-Store 2 [103].

Densitometer software such as CAMAG winCATS incorporates peak identity and purity checks based on in situ spectra comparison; identity checks compare spectra recorded at the maxima of sample and standard peaks, while purity checks compare spectra recorded at the start, middle, and end of a peak. However, it was suggested that spectral correlation cannot be considered a reliable measure of method specificity and that other chemometric methods are needed [137]. Based on later studies, PCA analysis [138] and self modeling multivariate curve resolution (non-negative matrix factorization) analysis [139] of densitograms of TLC spots were recommended.

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

6.1. Chemical detection

Changes in fluorescence emission due to noncovalent interactions were proposed as a general detection procedure for TLC [140]. A modified Dittmer-Lester spray reagent with added $SnCl_2$ was described for qualitative and quantitative phospholipid determination by TLC-image analysis [141]. Plasticizers in poly(vinyl chloride) commodities were quantified by silica gel HPTLC-densitometry after detection by dipping the plate into primuline solution and use of a newly introduced lipase treatment [142]. Six synthetic oligopeptides were detected on RP18 plates at the 7.5 ug level by dipping plates for 2 sec into 2% ninhydrin in ACT-HAc (25:1), heating at 80°C until characteristic yellow to pink/red zones appeared, and imaging the zones with a CAMAG TLC Visualizer [143]. Anisaldehyde-sulfuric acid reagent gave differently colored zones for the selective detection of sesquiterpene lactones and ecdysones from *Asteraceae* on silica gel plates [144]. Sensitivity and selectivity in the detection of estrogens were compared for four reagents: vanillin-phosphoric acid, manganese chloride-sulfuric aid, PMA, and ceric ammonium sulfate-phosphoric acid [145]. Strategy was described for optimization of the temperature and time during postchromatographic derivatization detection of ginkgolides and bilobalides using acetic anhydride with observation under 254 and 366 nm UV light [146]. Sixteen amino acids were detected with distinguishable colors and ng sensitivities on silica gel G plates using four new 0.05-0.25% ethanolic spray reagents comprising benzoic acid and its *p*-fluoro, -chloro, and -iodo derivatives [147]. A TLC plate containing fluorogenic naphthalimide functionalized SiO_2 nanoparticles prepared by intermolecular hydrogen bonding interactions served as a separation medium for Pb(II) ions from other metal ions and a fluorodetector [148].

6.2. Biological detection

Spangenberg reminded readers in an editorial in the Journal of Planar Chromatography (2013, volume 26, issue 5, page 385) that TLC is the only separation method that detects analytes in a dry stationary phase, which allows the use of biological systems for their detection because the dry layer interacts either with

enzymes or living cultures. He termed this kind of detection as activity analysis, often named EDA, and stated that he sees the challenges of modern TLC more in the field of activity analysis than classical detection. This view is strengthened by the fact that published papers involving biological detection have been increasing in each recent two year review period, and this trend is likely to continue in the future. Mechanisms and methodology of high throughput TLC bioautography (antimicrobial, antixoidant, and enzyme inhibition) techniques for screening of bioactive natural products [149] and concepts and technologies for tracking bioactive compounds in natural product extracts through generation of libraries and hyphenation of analytical processes, including TLC, with bioassays [150] were reviewed.

HPTLC was combined with the YES for the EDA of estrogenic compounds at pg LOQ levels; automated two step development of a silica gel 60 F plate with MeOH and then CHL-ACT-petroleum ether (PE) (55:20:25) was done with a CAMAG AMD 2 instrument [151]. Identification of ACE inhibitors from traditional Chinese medicinal plant seeds of the genus *Peganum* was made using silica gel plates with EtAc-MeOH-ammonia (10:1.5:0.5) mobile phase and detection by Dragendorff and vanillin-sulfuric acid reagents as well as by bioautographic assay [152]. Tylosin, spiramycin, and eryhtromycin residues were determined in Egyptian buffalo meat using extraction by 0.2% meta-phosphoric acid-MeOH (6:4), cleanup on Oasis HLB (hydrophilic-lipophilic balanced RP sorbent) 200 mg cartridges (Waters Corp.), silica gel 60 TLC with MeOH-EtAc-ACT (5:3:2) mobile phase, and immersion bioautography by the Michard and Joubert method [153]. TLC immunostaining was described for detection of anti-phospholipid antibodies in seronegative anti-phospholipid syndrome on silica gel 60 HPTLC aluminum plates preincubated with 1% potassium oxalate in MeOH-water (2:3) for 1 h, dried, activated at 100°C for 5 min, and developed with CHL-ACT-MeOH-HAc-water (40:15:13:12:8) [154]. Isolation and characterization of antibacterial chamomile ingredients were performed by use of direct bioautography against *Vibrio fischeri* luminescent marine bacterium, TLC and OPLC separations, isolation of active fractions by preparative layer chromatography (PLC), and solid phase microextraction (SPME)-GC/MS [155]. A TLC-bioautographic method was introduced for detecting lipase inhibitors in methanol extracts of *Camellia sinensis* L. and *Rosmarinus officinalis* L. in which after TLC migration of the extract the plate was sprayed with alpha-naphtyl acetate and enzyme solutions before incubation at 37°C for 20 min and spraying with Fast Blue B to give a purple background; lipase inhibitors were visualized as white spots down to 0.01 ug [156]. HPTLC-bioautography was used to separate and identify antioxidants extracted using Soxhlet, sonication, and maceration methods from *Piper betle* leaf, and then their amounts were determined by a new HPTLC-densitometry method that also served for the QC of leaf extract formulations [157]. A direct bioautography test of growth conditions for Gram-positive bacteria *Bacillus subtilis* was carried out on a TLC plate without mobile phase development; the preincubation and incubation times and temperature and the viscosity of the bacterial broth, the incubation time and temperature of the seeded plates, and the detection of spotted samples by spraying with 0.2% aqueous 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution followed by further incubation were optimized [158].

6.3. TLC/MS

A very high level of research in the techniques and applications of TLC coupled with MS [101] occurred in the past two years, and it is expected to increase in the future. Reviews were published on the analysis of phospholipids and glycolipids by TLC/matrix assisted laser desorption and ionization MS (MALDI-MS) [159]; MALDI imaging of lipid biochemistry in tissues [160]; application of different modes of TLC/MS for separation, detection, and qualitative determination of large and small biomolecules such as proteins, peptides, oligonucleotides, amino acids, DNA, RNA, and lipids [161]; silica gel TLC separation of glycosphingolipids, their overlay detection on the plate mostly based on antibody mediated recognition, and direct and indirect MS structural characterization, *i.e.*, directly on the plate or in lipid extracts from zones scraped from the plate [162]; recent advances in direct open air surface sampling/ionization [163]; and techniques and applications of the coupling of TLC/HPTLC with direct analysis in real time (DART) MS [164]. Online coupling of TLC and MS using a device that removes a desired spot completely by a plunger that is first pressed on the TLC plate to form a tight seal around the spot followed by solvent flow forced through the enclosed spot to extract it quantitatively was discussed in an article by Heinrich Luftmann [165]. This device was developed by Morlock and coworkers and eventually commercialized as the CAMAG TLC-MS Interface [108]. It was reported in February, 2013, that CAMAG and Advion Inc. formed an alliance to commercialize the TLC/CMS system combining the TLC-MS interface with Advion's expression compact mass spectrometer with a cost of around 48K Euros (amount provided by Morlock in a personal communation).

The following TLC/MS methods and analyses were reported in the review period: TLC/plasma assisted

multiwavelength laser desorption ionization (PAMDI)-MS integrated with DART for the facile separation and selective identification of low molecular weight compounds such as dye mixtures, drug standards, and tea extract with an LOD of 6 ng mm^{-2} on a silica gel layer [166]; a blotting method that transferred peptides separated on a wettable HPTLC plate to a hydrophobic RP C8 HPTLC plate was shown to be suitable for analysis with a liquid microjunction surface sampling probe ESI-MS system [167]; 1D and 2D HPTLC using silica gel 60 plates with a CAMAG Automatic Sampler 4 (ATS 4), AMD 2 developing chamber, and Immersion Device III to apply different staining solutions coupled with MALDI-TOF-MS for investigating polyphenol-protein interactions [168]; cellulose TLC/MS for screening of flavanols in the entire chromatograms from *Juniperus communis* L. and *Punica granatum* L. [169]; quantification of tissue globotetraosylceramides in a rat model of polyctstic kidney disease by silica gel HPTLC with CHL-MeOH-0.2% calcium chloride (50:35:8) mobile phase, spraying the plate with primulin, scraping off zones and eluting with MeOH, and MALDI-MS with automated data acquisition (indirect HPTLC/MS) [170]; determination of small, saturated hyaluronan oligosaccharides with HPTLC using reagent free derivatization by heating on amino modified silica gel layers and hyphenation with ESI-Q-TOF-MS [171]; rapid detection of haloarchael carotenoids *via* LLME enabled direct TLC/MALDI-MS in a simple bordering mode to limit sample diffusion during matrix addition on TLC plates [172]; HPTLC on silica gel aluminum plates developed with CHL-MeOH-90% HAc (65:4:35) mobile phase and use of multiple reagents to detect different lipid classes extracted by a modified Bligh and Dyer method from the hyperthermophilic archaeon *Pyrococcus furiosus,* followed by MALDI-TOF-MS after cutting the layer in pieces containing the lipids fractions and fixing them onto the MALDI target with tape [173]; analysis of drugs in postmortem biological samples using a silica gel TLC plate developed with ACT-28% aqueous ammonia mobile and then sprayed with 3 mL of 20 mg mL^{-1} alpha-cyano-4-hydroxycinnamic acid in 0.5% trifluoroacetic acid (TFA)-ACN (3:7) and fixed in a plate holder for MALDI-MS [174]; frontal elution with a strong solvent of a powder sample (herbal medicine, pig feed, or house dust) placed directly on a V-shaped paper strip to extract and develop the test analytes so they condense at the tip, which was then placed under the visible plasma for desorption corona beam MS analysis [175]; construction of a "green" interface for high throughput TLC and ambient electrospray assisted laser desorption ionization (ELDI)-MS analysis comprising a storage box, plate introduction component, conveyer, light sensor, and plate collection box as building blocks was demonstrated and applied to analysis of drugs and dyes [176]; monitoring of chemical transformations by direct combination of TLC with nanoparticle assisted laser desorption/ionization MS [177]; and quantificaion of anthocyanes in pomace, feed, juice, and wine using silica gel 60 F plates, two different mobile phases for determination of anthocyanins and anthocyanidins, and ESI-MS coupled by the CAMAG TLC-MS interface [178].

Reagent free derivatization mentioned above [171] is a well known detection method in which a developed amino plate is heated to form fluorescent zones of certain analytes (*e.g.* sugars) through a thermochemical reaction without application of a detection reagent. In 2001 my laboratory extended this detection approach for the determination of creatine in nutritional supplements by heating a silica gel F plate on a CAMAG plate heater at 160°C for 5 min to form a fluorescence quenching zone. No other reference to this detection method has appeared to my knowledge since we reported it, but now we have used it for detection of artesunate and artemether in their silica gel HPTLC-densitometry determination in tablet formulations; fluorescent quenching zones of the drugs were formed by heating on a silica gel F plate without the need for the usual methanol-sulfuric acid spray detection reagent (results to be published in 2014). Thermochemical reaction on silica gel F plates to form fluorescence quenching or fluorescent zones may be a quite ubiquitous effect that can be utilized in place of postchromatographic detection reagents in TLC (artesunate and artemether zones also fluoresced, but better results were obtained by scanning at 254 nm than 366 nm).

6.4. TLC Coupled to Other Spectrometric Methods

TLC is easily and often combined with UV-Vis spectrometry by use of a modern densitometer such as those from CAMAG (TLC Scanner 3 or 4) and Desaga (CD-60) in the *in situ* spectral mode. Zone identity is usually based on comparison of spectra of samples with those of standards in a spectrum library option of the densitometer software.

Peak purity assessment and identification of drugs in multicomponent mixtures were carried out based on the use of the derivative profile of the densitogram and the derivative ratios as fingerprints of the compounds; the wavelengths of absorbance and derivative (first through fourth) optima of the extracted densitograms were allocated and the data compared with those obtained using the corresponding reference standards [179].

Plates were prepared from non-infrared (IR) absorbing AgI fine particles for the coupled TLC-Fourier transform IR (FTIR) anaysis of rhodamine B

and bromophenol blue as model compounds [180]. Plasma jet desorption atomization-atomic fluorescence spectrometry using an argon dielectric barrier discharge plasma jet generated inside a 300 um quartz capillary was coupled online with TLC for speciation of Hg^{2+}, methylmercury, and phenyl mercury dithiazonates with LOD as low as 8.7 pg [181]. The potential of the CAMAG TLC-MS interface for coupling TLC to nuclear magnetic resonance (NMR) spectrometry was demonstrated for identification and quantification of active ingredients in pharmaceutical formulations and plant extracts [182]. A method integrating TLC with femtosecond laser ablation inductively coupled plasma (ICP)/MS was developed for direct determination of the proportionality of V/Ni in the asphaltene fraction of crude oils on the plate without any additional sample preparation [183].

TLC was coupled to SERS [112] in the following studies: use of silver nanorod array substrates for on-chip separation and detection of test dye mixtures by combining UTLC and SERS [184]; a method for hyphenating SERS and TLC that employs silver polymer nanocomposites as an interface through the process of conformal blotting [185]; preparation of a SERS active silver substrate under open air conditions directly on a thin silica film by photoreduction of silver nitrate for detection of binary mixtures based on a unique spectral fingerprint for each molecule [186]; separation and identification of the alkaloids harmalol, harmaline, harmane, and harmine from seed extract of Syrian rue by separation on a silica gel HPTLC plates, detection under UV light, and deposition of 0.8 uL of Ag colloid and 0.1 uL of aqueous 0.5 M potassium nitrate on top of each spot followed by SERS while still wet [187]; and separation and detection of target analytes from complex samples (*i.e.*, melamine in infant formula and heroin in samples contaminated with a highly fluorescent background) using 2D chromatographic separation on inkjet printed paper SERS substrates (silver nanoparticle ink preparation and lateral flow concentration after TLC and prior to SERS measurement are described) [188].

7. Quantitative analysis

Modern quantification in TLC usually involves the use of a slit scanning densitometer after sample preparation; automated application of standards and samples to the layer usually as bands using a CAMAG Linomat, CAMAG ATS 4, or Desaga AS-30 (zones in the shape of bands can be better resolved than round spots); mobile phase development of the layer in a CAMAG TTC, CAMAG ADC 2, or CAMAG or Chromdes horizontal chamber; and detection of the separated analyte zones. A densitometer such as the CAMAG Scanner 3 scans a series of standard zones; a calibration curve is generated from the peak height or area *versus* weight data by linear, nonlinear, polynomial, or Michaelis-Menten regression; and the weights of bracketed sample zones are interpolated from the curve. Internal standardization is only occasionally used in TLC-densitometry, most commonly in clinical drug analyses, *e.g.* quantification of propanolol in human serum [189]. Use of image analysis [141] (*e.g.* camera-based densitometer, videodensitometer, and office-type computer scanner densitometer) is being reported to an increasing degree, and selected examples are described below.

7.1. Techniques and instruments

TLC/HPTLC quantitative methods must be validated to prove the reliability of the obtained results. Spangenberg described how LOD and LOQ can be measured based on signal to noise ratio or the standard deviation (SD) of the response and slope to satisfy International Conference on Harmonization (ICH) validation guidelines, by the EURACHEM approach, and by the calibration curve approach of Andre Habaux and Gilbert Vos [190]. Correct calibration in planar chromatography was discussed by Rudolph Kaiser, a world recognized expert in the field [191]. The importance of standardization in quantitative TLC based on automation of the main analytical steps and application of operational qualification and performance qualification (OQ/PQ) was illustrated using experimental results with a CAMAG ADC 2 in the quantification of the pigments lutein and beta-carotene [192]. A strategy was given to minimize methodological failures in HPTLC method development and validation [193]. The use of accuracy profiles was proposed as an alternative approach to classic validation of HPTLC methods for assay of drug formulations [194].

TLC with image analysis was used in the following studies: determination of nonchromophoric tuberostemonine alkaloid derivatives in *Stemona* species using silica gel 60 F plates, DCM-EtAc-MeOH-ammonum hydroxide (50:45:4:1) mobile phase, postchromatographic derivatization by dipping into Dragendorff reagent, and image analysis with a digital scanner (Hewlett Packard Deskjet F370) and evaluation of the image file using CAMAG VideoScan software [195]; determination of association constants of charge transfer complexes between N,N-diethylaniline and nitro explosives using TLC with scanning videodensitometry [196]; automatic lane detection in TLC images [197]; investigation of antiradical substances in plant material by observing and recording the bleaching of the purple DPPH color on a layer by these substances with a photo

camera and then making quantitative measurements using the free, open access ImageJ processing program (developed by the U.S. National Institutes of Health) [198]; quantification of sibutramine in adulterated herbal slimming formulations using silica gel F plates developed with TOL-HEX-diethylamine (DEA) (9:1:0.3), Dragendorff reagent for spot detection (dip application with a CAMAG Immersion Device 3), and image analysis by Sorbfil TLC videodensitomer software after digital scanning of the plate (Hewlett Packard SCAN Jet 3500C) [199]; monitoring of sonolytic degradation of surfactants in wastewaters on silica gel plates with image acquisition, processing, and archiving controlled by CAMAG VideoStore documentation software and VideoScan software for quantitative evaluation [200]; a new UV-Vis TLC scanner device and three different softwares were used for quantitative determination of ethyl and butyl parabens in pharmaceutical suspension, *i.e.*, ImageDecipher TLC, Sorbfil TLC, and JustTLC, and the first software gave the best results [201]; TLC with videodensitometry as a detection technique and an artificial neural network (ANN) to analyze chromatograms as fingerprint patterns for quantification of three phenylpropanoid markers in commercial Echinacea products [202]; polyphenolic compounds isolated from *Medicago sativa* and *Medicago truncata* were tested for free radical scavenging activities with a rapid TLC dot-blot test, and active ones were further chromatographed on silica gel plates using ACN-water-CHL-HCOOH (60:15:10:5) mobile phase, staining with DPPH* methanolic solution, and quantitative measurement with an improved ImageJ processing protocol [203]; validation of a densitometric method for determination of theanine in tea extracts using silica gel F plate development with *n*-BuOH-ACT-HAc-water (7:7:2:4), ninhydrin detection reagent, photography of plates with a Fujifilm digital camera, and analysis of the green channels of the photograph by free CP Atlas 2.0 software using the dark on light option (http://www.lazarsoftware.com) [204]; quantitative monitoring of the progress of two organic reactions (alkaline hydrolysis of phenyl benzoate and reduction of benzaldehyde derivatives) by multivariate image analysis after TLC (silica gel layer with HEX-EtAc 15:1 and 3:1 mobile phases) with a homemade imaging cabinet and a homemade program to convert image data into three and two dimensional chromatograms from which changes in the amounts of reactants as a function of time were evident [205]; determination of valsartan and amlodipine in a combined dosage form by C8 F TLC with ACN-phosphate buffer pH 9 (1:1) mobile phase and videoscanning at 254 and 366 nm, respectively, with a Desaga VD40 video system and a Mitsubishi color video CCD (charge couple device) camera Model CP 700D [206]; and use of an Epson Perfection V500 Photo Scanner both at 300 and 600 dpi resolutions in 16 bit TIFF greyscale image format and image analysis with gel analysis TL 120 software to determine percentages of lipid fractions of egg yolk separated on a silica gel plate and detected with 10% cupric sulfate-8% phosphoric acid charring reagent [207].

Separations of samples applied in nL volumes were recorded on Merck UTLC plates in full color with 32 um spatial resolution and 33 msec temporal resolution using a newly designed measurement system. The code written for MATLAB Version 7.8 R2009a (MathWorks Inc.) analyzes multiple tracks per plate, filters analyte spots by color, and automatically generates time resolved figures of merit. Although Merck UTLC plates are no longer commercially available, the methods described may lead to improved analytical performance on new UTLC plates being prepared in various research laboratories (see Section 4.1) [208].

It is expected that use of quantitative densitometry on electronic images using software based evaluation will increase in the future compared to slit scanning densitometry because of much lower cost and ease of operation. However, image analysis does not possess the spectral selectivity offered by a densitometer with a monochromator, and the software used must have the ability to verify that no editing of the original image has occurred if analyses must be performed in compliance with GMP/GLP requirements (Desaga ProVidoc/ProQuant and CAMAG winCATS/VideoScan softwares have this feature allowing assurance of the absence of image alteration). Image analysis is ideal for comparison of chromatograms within a plate and between plates and for visual identification purposes, *e.g.* in medicinal plant fingerprinting. The scanner or camera used to obtain the image must have a UV emitting source or only colored zones, and not those that quench fluorescence or are fluorescent, can be imaged and evaluated.

7.2. Applications

In addition to the very many quantitative analyses already cited in previous sections of this review, this section presents selected examples for a variety of analytes and sample types for which new or improved densitometic methods were reported in the review period, with validation information. Unless otherwise noted, Merck silica gel 60 F glass or aluminum plates, a CAMAG automated applicator, ascending development in a TTC, and a slit scanning densitomer in the reflectance-absorption mode were used in the methods. The vast majority of TLC-densitometry analyses reported in the literature are for pharmaceutical bulk drug and dosage

forms, and for plant samples and herbal medicine and nutritional supplement formulations.

HPTLC analysis of salicylic acid in release medium during development of adhesive topical skin care antiacne patches was performed with TOL-MeOH-HAc (74:25:1) mobile phase and scanning at 270 nm. Linearity was in the range of 57-399 ng per zone with a correlation coefficient (r) of 0.999; LOD and LOQ were 25 and 57 ng per zone, respectively; relative standard deviation (RSD) for repeatability and intermediate precision were less than 4.0%; and recovery based on analysis of spiked simulated samples was 96.8-103% [209].

The bactericide bacitracin was quantified in antibiotic ointment that also contained neomycin sulfate by extraction with pH 9 carbonate buffer, derivatization with dabsyl chloride, development in a glass N-chamber with *n*-BuOH-2-butanone-25% ammonia-water (10:5:2:2), and scanning at 460 nm. LOD was 8.6 ng/band, LOQ 26.2 ng per band, linearity 85.4-853.6 ng per band, and RSD 1.23-2.47%, and accuracy was validated by comparison of results with a recognized differential spectrometric method [210].

Many drug assay development studies include proof that the method is stability indicating in the presence of degradation products formed under ICH recommended stress conditions. For example, the antidepressant fluvoxamine was determined in bulk drug and a tablet formulation using EtAc-TOL-MeOH-ammonia (7:2:1:0.5) mobile phase and scanning at 254 nm. The drug was subjected to acid and alkali hydrolysis, oxidation with H_2O_2, and sunlight and UV light photodegradation, and peaks of degradation products were well resolved from that of the pure drug. Data for calibration curve linearity, precision (interday and intraday), LOD, LOQ, robustness (effect on results of small, deliberate variations in method parameters), specificity (peak purity), and accuracy (recovery using the standard addition method) were all within ICH guidelines [211].

The drug hydrocortisone acetate and preservative 2-phenoxyethanol were simultaneously determined in a pharmaceutical cream using extraction with 96% ethanol, C18 F aluminum plates developed with MeOH-water (22:8), and scanning at 270 nm. Respective validation data were linearity 4.26-13 and 0.75-4.25 ug per spot, LOD 554 and 149 ug per spot, LOQ 1660 and 448 ug per spot, and RSD <2% [212].

The antidepression drug duloxetine was determined in pH 11 carbonate buffered human serum using vortex extraction with HEX-isopropanol (iso-PrOH) (97:3), ACT-benzene-diethylamine (DEA) (5:4.5:0.5) mobile phase, and scanning at 235 nm. The calibration curve was linear from 35-140 ng per spot (r^2 0.980); LOQ 35 ng per spot; LOD 10 ug per spot; intraday and interday RSD 1.83 and 5.66%, respectively; and recovery 92.9-97.6% [213].

A validated HPTLC method was described for quantification of the anticancer agent betulinic acid (BA) from two Indian plants of the species *Dillenia* and *Ziziphus*. After MeOH reflux extraction and TOL-CHL-EtOH (4:4:1) mobile phase development, anisaldehyde sulfuric acid reagent detected BA as a magenta colored zone that was densitometrically evaluated at 526 nm. Validation according to ICH guidelines was performed. BA was found to be highest in *Dillenia indica* bark at 0.43% [214].

Commercial whitening creams containing the phenolic glycoside arbutin that is present in the leaves of several plants were analyzed by hot methanol extraction of defatted samples, MeOH-CHL-HAc (3.5:6:0.5) mobile phase, and scanning at 285 nm. Validation was carried out under ICH guidelines, and LOD and LOQ were 42.2 and 112 ng per spot, respectively. Stability was evaluated under acid and base hydrolysis, oxidation, and thermal stress conditions [215].

The flavonoids diosmin and hesperidin were simultaneously determined in lemon, orange, and tangerine and a tablet formulation. Ultrasonic extraction of samples was with MeOH-DMSO (1:1), the mobile phase was EtAc-MeOH-water-HAc (25:2:2:1), and scanning was at 330 nm. Full validation was based on ICH guidelines, with respective calibrations linear in the 0.1-3 and 0.25-7.5 ug per spot ranges [216].

The antituberculosis compounds baicalein, hispidulin, chrysin, and oroxylin A from the Thai vegetable *Oroxylum indicum* used in the Indian Ayurvedic system of medicine were quantified by percolation extraction with MeOH, solvent partitioning and silica gel column cleanup, CHL-MeOH-water-HCOOH (97:3:0.5:0.5) mobile phase, and scanning at 270 nm. Full ICH validation was carried out, and recovery of the four compounds in standard addition analyses ranged from 95.2 to 100.2% [217].

Glabridin, a stable flavonoid of *Glycyrrhiza glabra*, was determined in licorice rhizome and a polyherbal formulation using reflux extraction with 70% EtOH, TOL-DCM-EtAC (1:1:1) mobile phase, and scanning at 287 nm. Validation was based on ICH guidelines, and stability was tested with acid, base, oxidation, thermal, humidity, sunlight, and UV light stress. Recovery was 97-103% in standard addition experiments at 0, 50, 100, and 150% of the theoretical value [218].

A method comprising CHL extraction by sonication and shaking, DCM-MeOH (28:15) mobile phase, and scanning at 254 nm was used to determine nicotine content in different brands of cigarettes. The method

was validated for all ICH parameters, and accuracy was 97.5 and 98.4% and RSD 2.43 and 3.19% for standard addition recovery experiments at two spiked levels [219].

The vitamin K homologs phylloquinone, menaquinone-4, and manaquinone-7 were simultaneously determined in tablets and ampoule formulations and spinach, cabbage, pear, and apple samples by scanning at 254 nm after plate development with MeOH-EtOH-iso-PrOH-water (75:5:5:15). The pharmaceutical formulations were extracted by sonication with EtOH and the fruits and vegetables with HEX. Respective R_f values were 0.56, 0.43, and 0.23; linearity was 2-200 ng per band (r = 0.999) for the three calibration curves; LOD was 0.19-0.85 ng per band; and LOQ was 0.76-2.5 ng per band. Validation was performed in accordance with ICH guidelines, and results of the vegetable and fruit analyses compared closely to those obtained using an earlier HPLC method [220].

Toxicological characterization of *Alternia alternata* strains isolated from wheat (34 strains) and grape (11 strains) and then cultured on rice (incubated in the dark at 25°C for 21 days) was carried out by HPTLC-densitometry quantification of the mycotoxins alternariol (AOH), AOH monomethyl ether (AME), altenuene (ALT), and L-tenuzanoic acid (TeA) in the cultures. The mycotoxins were extracted by blending with MeOH and ammonium sulfate, and extracts were cleaned up by LLE with DCM. Slica gel 60 F layers impregnated with oxalic acid in MeOH were used for the separation with TOL-EtAc-HCOOH (6:3:1) mobile phase in a CAMAG Horizontal Developing Chamber. Chromatograms of ALT, AOH, and AME were scanned in the fluorescence mode with 254 nm excitation and a 400 nm measuring filter; chromatograms for determination of TeA were sprayed with $FeCl_3$ in ethanol, and the detected spots were scanned in the absorbance mode at 254 nm. Respective R_f values were 0.25, 0.36, 0.49, and 0.30; recovery (mass fraction) was 0.59-0.76 in the 20-100 mg kg^{-1} range in rice cultures; RSDs of repeatability were 7-19%; and LOQ was 1.0 mg kg^{-1} for ALT, AOH, and AME and 5.0 mg kg^{-1} for TeA in rice cultures with *Alternaria alternata* mycelium [221].

8. PLC

Traditional PLC is carried out on precoated layers with 1 or 2 mm thickness to obtain increased sample loading capacity by applying bands of sample across the layer. This form of PLC is seldom reported now in the literature, but “micropreparative layer chromatography (MPLC)” using thinner layers (*e.g.* 0.20, 0.25, or 0.50 mm) with larger sample sizes than used in analytical separations but not as large as in traditional PLC is often reported. The plates usually contain an F indicator to facilitate zone detection. A few applications of PLC are cited in this section as will a a new stationary phase for rotational PLC.

The lipid components of the total lipid extract of *Pyrococcus furiosus* were separated by MPLC using 0.2 mm thick silica gel layers on aluminum backing with CHL-MeOH-90% HAc (65:4:35) mobile phase. Lipids were visualized by staining with iodine vapor and were eluted from scraped silica gel for further analysis [173].

Crude extracts of selected plants were subjected to column chromatography and the resulting fractions tested for antibacterial activity toward organisms such as methicillin resistant *Staphylococcus aureas*. The bioactive column chromatography fractions were further separated by PLC on preparative silica gel 60 F plates developed with EtAc-PE (40-60°C) (4:6) or DCM-MeOH (98:2-95:5, depending on the polarity of the analytes), and the resulting bands were investigated by HPLC/ESI-IT-MS [222].

In a study to optimize an extraction and separation procedure to obtain a concentrated fraction with antibacterial activity from macroalga, eight solvents were tested for extraction of antibacterial compounds, bioautography was used to screen crude extracts, and antibacterial compounds were first separated from the crude extract by PLC on Analtech preparative TLC plates with sample application across the full width. One of the four visible fractions was active, and it was scraped from the layer and eluted with EtAc [223].

Profiles of triacylglycerols (TAGs) isolated from three bat species were determined by MALDI-TOF-MS. Neutral lipids extracted from the hair and wing tissue were fractionated by PLC into bands of sterols, free fatty acids, TAGs, and sterol wax esters, and the TAG band was recovered for MS analysis. Silica gel plates with a 0.2 mm layer thickness were spotted with a CAMAG Linomat 5, developed with HEX-DEE-HAc (80:20:2) mobile phase, sprayed with 0.05% rhodamine 8G in 95% ethanol, and viewed with a UVP fluorescent docking station. Silica gel containing the TAG fraction was scraped and eluted with CHL-MeOH (3:2), and the eluate was analyzed by MS. TLC-densitometry after zone detection with 3% cupric acetate in 8% phosphoric acid reagent showed higher proportions of TAGs in hair *versus* wing tissue [224].

A simple, fast, and efficient HPTLC method was developed for simultaneous quantification of alcohols and acetates in Haitian vetiver essential oils and vetiver acetates by HPTLC separation on silica gel using HEX-CHL-EtAc (8:6:0.5) mobile phase at 47% relative humidity and densitometry at 530 nm after detection

with vanillin-sulfuric acid reagent. Reference mixtures of alcohols and acetates were obtained by fractionation of the oil or acetates followed by purification of the fractions of interest by preparative OPLC using an OPLC 50 system with 20×20 cm silica gel 60 F HTSorb OPLC chromatosheets (11 um particle size) sealed on four sides and positioned in the PTFE covered cassette inserted into the OPLC separation chamber. Determination of the chemical compositions of each fraction was done by GC/MS or GC×GC/MS [225].

Centrifugal or rotational PLC is carried out on commercial Chromatron or Analtech CycloGraph instruments. A method for preparation of RP silica gel rotors for these instruments was developed and their application was described for isolation of the diastereoisomeric alkaloids banistenosides I and II from *Banisteriopsis*, saponins III and IV from *Fagonia cretica*, and the sesquiterpenes atemisinin (V) and artemisinic acid (VI) from *Artemisia annua* [226].

9. Thin Layer Radiochromatography (TLRC)

TLRC (also called radio-TLC) is essentially overlooked in books and review articles on planar chromatography, but it is a very important and widespread application to separate, detect, and quantify radioactive zones on thin layer and paper chromatograms. More than 45 research papers on this technique were collected in the review period, published in dedicated radioanalytical, nuclear chemistry, and nuclear medicine journals, as well as general pharmacy, medicinal, biology, and biochemistry journals. The methodology described in these papers almost never involves automated sample application or mobile phase development but more traditional techniques, with detection and quantification by specialized methods such as autoradiography, scintillation counting, phosphor screen imaging, and in situ radioscanning. The most used stationary phase reported is Instant Thin Layer Chromatography (ITLC)-SG binderless glass microfiber chromatography paper sheets impregnated with silica gel. Formerly a product, in turn, of Gelman Sciences, Pall Corporation, and Varian Corp., ITLC sheets are now available from Agilent Technologies. Many of the published papers contain sparse experimental detail, e.g., not stating the mobile phase and/or stationary phase used, how the samples were prepared and applied, and/or how the separated spots were detected. The major use of TLRC is verification of radiochemical purity (QC) for nuclear medicines, but other studies involving radiolabeled compounds are also published as illustrated below.

A new method for radiochemical purity measurement of ^{111}In-pentetreotide (OreoScan) used for diagnosis and management of carcinoid tumors bearing somatostatin receptors replaced the 0.01 N sodium citrate (pH 5) mobile phase used to develop ITLC-SG sheets in the standard method with ACD-A. This solution contains dextrose monohydrate, sodium citrate, and anhydrous citric acid and is commonly available in radiopharmacy units for use as an anticoagulant agent when cells are labeled with radionuclides, so it does not have to be prepared before each TLC analysis. The distribution of ^{111}In on the strips was measured by a Raytest Mini-Gita radioscanner with an NaI detector and window between 200 and 300 keV [227].

Urinary radioactivity was characterized by TLC in a study of the disposition of ^{14}C-AHTN and ^{14}C-HHCB, polycyclic musks widely used as fragrance ingredients in consumer products, after intravenous administration to Sprague-Dawley rats and domestic pigs. Urine was applied to a silica gel 60 F plate with 0.26 mm layer thickness as 2 cm wide bands, and the plate was developed with CHL-MeOH-ammonia (80:20:1). Urinary components presumed to be metabolites were detected and quantified using a Fuji Photofilm Co. Fujix BAS 2000 phosphor bioimaging analyzer employing Raytek Scientific Ltd. PC-BAS 1000 software [228].

Synthesis of ^{90}Y- and ^{177}Lu-radiolabeled somatostatin analogs for treatment of tumors was described, and their radiochemical purity was assessed using C18 F plates developed with 1 M ammonium acetate-MeOH (1:1) and ITLC-SG strips developed with 0.1 M sodium citrate-1 M HCl (97:3). Autoradiography chromatograms were illustrated to indicate the purities obtained [229].

Photoreduction of ^{99}Tc pertechnetate by nanometer sized metal oxides for formation and sequestration of low-valent Tc was monitored as a function of time using Whatman 3MM CHR paper strips and 0.9% saline mobile phase. Quantification was automatically performed on a Bioscan AR-2000 imaging scanner, providing the percent of total activity of each peak. The Bioscan AR-2000 is probably the most widely used detection/quantification instrument in TLRC research today [230].

Methanol extracts of yarrow plants were purified, labeled with ^{131}I, and used for biodistribution studies in Balb/C mice. QC of the radiolabeled bioactive components was done using cellulose plates, *n*-BuOH-EtOH-0.2 M ammonium hydroxide (5:2:1) mobile phase, and counting of plates with a Bioscan AR-2000 [231].

Bleomycin-glucuronide was labeled with ^{131}I and used in biodistribution studies with a xenograft model of human colon tumor in Balb/C mice. For QC studies, a 20×20 cm cellulose aluminum plate was developed

with a solution of citric acid monohydrate, the plate was covered by an adhesive band (cello-band) and cut into 0.5 wide pieces, and each piece was counted by using a Cd(Te) detector equipped with a RAD 501 single channel analyzer [232].

Anti-carbonic anhydrase IX monoclonal antibody was labeled with ^{125}I for preliminary biological evaluation in mice bearing HT-29 tumors. The radiochemical purity of the antibody was determined on silica gel 60 G F plates using BuOH-pyridine-HAc-water (30:20:6:4) mobile phase and a Bioscan AR-2000 with Winscan version 3.09 software [233].

The reactions of ^{99m}Tc of different oxidation states with 2-thiouracil and 5-nitrobarbituric acid were studied at different temperatures, pH values, and concentrations. Reaction mixtures were monitored at different times using TLC on silica gel plates (250 um layer) with ACN-water (95:5) mobile phase and a Bioscan AR-2000 to detect the radioactive spots and determine the percentage of the activity of each [234].

Radiochemical incorporation yields in the microfluidic labeling of ^{18}F-altanserin were examined on silica gel 60 F plates developed with ACN-water (8:2). Measurement of radioactive spots on the plate was done by "electronic autoradiography" with a Canberra-Packard Instant Imager microchannel plate analyzer [235].

The importance of steric effects on the efficiency and fidelity of transcription by T7 RNA polymerase was evaluated by measuring the composition of RNA transcripts by enzymatic digestion (ribonuclease I) followed by 2D TLC. The reaction mixture was spotted onto a silica gel HPTLC plate that was developed by isobutyric acid-ammonia-water (66:1:33) in the first dimension and sodium phosphate (0.1 M, pH 6.8)-ammonium sulfate-PrOH (100:60:2, v/w/v) in the second. Quantification was performed employing a Molecular Dynamics PhosphorImager with ImageQuant software [236].

Paper chromatography on Whatman 3MM and 31ET strips and minicolumn chromatography using silica Sep-Pak cartridges were compared as an alternative for ITLC-SG in the radiochemical purity testing of ^{111}In-capromab pendetide. The 31ET was preferred based on slightly higher correlation to ITLC-SG, slightly tighter SD and range, and slightly shorter development time [237].

In a study of the pharmacokinetics of ^{18}F-flutemetamol in wild type rodents and its binding to beta-amyloid deposits in a mouse model of Alzheimer's disease, metabolism and binding to plasma proteins *versus* ^{11}C-Pittsburg compound B control were analyzed using TLC on C18 W plates with ACN-water (1:1) mobile phase. Using a Fuji Analyzer BAS-1800, the dried plates were exposed to an imaging plate for two physical half-lives of each tracer. The plate was scanned, and the proportions of nonmetabolized to metabolized tracer were calculated with Raytest Aida Image Analyzer, version 4.19 [238].

Suggestions for improvement of TLC in isotopic analysis were given based on quantitative spotting, data acquisition via digital color photography, quantification via ImageJ public domain image processing software, and application of a simple mathematical transform to linearize the raw data. Examples of use in some isotopic labeling applications were given [239].

In examples of other reported TLRC studies, ITLC-SG sheets were used in the radiochemical purity determination of radiopharmaceuticals from cold kits [240] and of peptides and antibodies radiolabeled with ^{68}Ga, ^{177}Lu, and ^{131}I using an automated module [241]; phosphor bioimaging was used to study the metabolic fate of ^{14}C-labeled nicotinamide and adenine in germinating propagules of the mangrove plant *Bruguiera gymnorrhiza* [242] and for radiometabolite analysis of ^{11}C biochemical partitioning to non-structural carbohydrates for integrated metabolism and transport studies [243]; and a Bioscan AR-2000 was used in the QC of synthesized ^{99m}Tc-D-penicillamine glucuronide [244], QC of ^{99m}Tc labeled diethylstilbesterol phosphate derivative [245], yield determination in the radiolabeling of epigallocatechin from green tea [246], incorporation level evaluation of ^{18}F into 2-(5-fluoro-pentyl)-2-methyl malonic acid potential PET (positron emission tomography) imaging agent [247], measurement of coupling efficiency of micelles with radioisotope ^{111}In in the preparation of a novel targeted nuclear imaging agent for gastric cancer diagnosis [248], and radiochemical purity analysis of ^{64}Cu-DOTA-trastuzumab for targeting ErbB2/Neu expression [249].

10. Conclusion

It has been shown in this review that TLC continues to be a very valuable and widely used analytical method that is applicable for virtually all analyte and sample types. The most significant research in techniques, materials, instrumentation, and applications during the past two years have been referenced, and future prospects are given in many sections throughout the review. Silica gel 60 F plates with 1D capillary action ascending or horizontal development at laboratory temperature are most widely used by far, and this is expected to continue with use of other chemically bonded and nonbonded layers and different development methods only when resolution levels not available under these conditions are needed and can be

achieved. Among the most active research areas that will continue to grow are TLC-densitometry, retention/lipophilicity studies, production of new UTLC layers, use of biological detection methods, and MS-TLC. The most prominent application areas will include pharmaceutical products [250], plant material active ingredients and their medicinal and dietary supplement formulations, foods and beverages, environmental samples, and radiochemical purity of labeled drugs. Development of miniaturized systems and "green" analyses will grow. Advantages of TLC are also mentioned throughout the review, such as less needed sample cleanup because of the use of fresh stationary phase in each experiment, and having complete chromatograms of multiple standards and samples on adjacent lanes of a single plate (high throughput) with the ability to use a variety of detection and quantification methods on each chromatogram.

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