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Review

Thin-layer chromatography: challenges and opportunities

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Abstract

The purpose of this article is to identify core technologies with the potential to influence the development of thin-layer chromatography over the next decade or so. Core technologies are identified as: (i) methods to provide a constant and optimum mobile phase velocity (forced flow and electroosmotically-driven flow), (ii) video densitometry for recording multidimensional chromatograms, (iii) in situ scanning mass spectrometry, and (iv) bioactivity monitoring for selective detection. In combination with two-dimensional, multiple development and coupled column–layer separation techniques these core technologies could dramatically increase the use of thin-layer chromatography for the characterization of complex mixtures. It is also demonstrated that thin-layer chromatography has strong potential as a surrogate chromatographic model for estimating biopartitioning properties. To convert these opportunities into practice the current state-of-the-art of the core technologies is described and the principle obstacles to progress identified.
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Keywords: Thin-layer chromatography; Reviews; Planar chromatography

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1. Introduction

In earlier reviews this author provided an overview of thin-layer chromatography at the turn of the century [1] and a separate account of method development for thin-layer chromatography [2]. In addition to these articles, Nyiredy edited a book providing a retrospective view of thin-layer chromatography for the third millennium [3]. These resources provide a contemporary picture of thin-layer chromatography as practised today.

Connected with symposia to celebrate the beginning of a new century for chromatography, this author presented a number of accounts of the prospects for thin-layer chromatography as a component of the collection of separation tools used in analytical laboratories. Common sense dictates that attempts at ‘‘crystal ball gazing’’ are better left to after hours socializing and should never be committed to print, lest we realize just how wrong we can be in predicting even the near future. That being said, any sensible predictions must have a basis in today's knowledge, and if considered as an extension of what has been achieved already, then it may not be too dangerous to propose what might be around the corner. Such predictions require identification of present deficiencies in a technique as well as deficiencies related to what is available for those techniques considered likely competitors for the principal applications of thin-layer chromatography. If a more convenient or reliable method for a separation appears then that method will be adopted and diminish the use of the technique it displaces. The reader will recognize that this has been a general trend with respect to thin-layer chromatography, which has continued to lose ground to high-pressure liquid chromatography in laboratory practice.

In the author's opinion thin-layer and high-pressure liquid chromatography should be viewed as complementary techniques that can be distinguished by their different attributes, resulting in a preference for one approach over the other on a problem by problem basis [1,4,5]. However, a limited appreciation of the attributes of thin-layer chromatography means that it is often overlooked for just those applications it would be most useful. Adding to the problem is that many laboratories no longer possess equipment for instrumental thin-layer chromatography and laboratory staff lack training in its use. These features have conspired to make thin-layer chromatography an invisible technique in the eyes of many experienced scientists. This article is not written to remedy this problem directly, but if thin-layer chromatography can be made more appealing through enhancing its capabilities, then its attributes will be more clearly recognized and its general use increased.

My attempts at ‘‘crystal ball gazing’’ led to a list of desirable features for advanced applications of thin-layer chromatography summarized in Table 1. Their implementation requires advances in core technologies, identified as external control of the mobile phase flow-rate, video imaging, interfaces for mass spectrometry, modeling and biomonitoring. With the exception of retention modeling (computer-assisted method development), dealt with elsewhere [2], these topics form the basis of this review.

2. Effective use of thin-layer chromatography

The attractive features of TLC include parallel sample processing for high sample throughput; accessibility of the sample for post-chromatographic

Table 1
The way ahead for thin-layer chromatography

- Pneumatic or electroosmotically-driven flow TLC with continuous on-line detection or image analysis
- Two-dimensional TLC with in situ image analysis
- TLC with in situ mass spectrometry for identification
- Pneumatic flow or electroosmotically-driven flow automated multiple development
- Optimized layers for biopolymer separations
- Structure-driven computer-aided method development
- Bioactivity detection for quality assessment
- Source of surrogate models for estimating biopartitioning properties

evaluation free of time constraints; detection in the presence of the stationary phase (is somewhat) independent of mobile phase properties; and the stationary phase is normally used once only. It is generally agreed that thin-layer chromatography is most effective for the low-cost analysis of samples requiring minimal sample clean-up, or where thin-layer chromatography allows a reduction in the number of sample preparation steps (e.g. the analysis of samples containing components that remain sorbed to the stationary phase). Thin-layer chromatography is also preferred for the analysis of substances with poor detection characteristics requiring post-chromatographic treatment for detection. Since all sample components are located in the chromatogram, thin-layer chromatography is the most suitable technique for surveying sample properties.

Many of these advantages are realized in large-scale surveillance programs. Examples include the identification of drugs of abuse and toxic substances in biological fluids [6–9], the identification of unacceptable residue levels of drugs used to prevent disease or promote growth in farm animals [10–13], maintaining a safe water supply by monitoring natural and drinking water sources for crop-protecting agents used in modern agriculture [14,15], and confirmation of label claims for content of pharmaceutical products [16]. For these applications, thin-layer chromatography is often used as a component of a pyramid strategy. This requires an efficient screening step (thin-layer chromatography) to identify suspect samples and a confirmation step (the most suitable analytical method) to establish the contaminant level in suspect samples. The benefits of this strategy are lower costs and an increase in the number of samples processed, allowing more violated samples to be identified [12]. Thin-layer chromatography is selected for the screening step because: (1) single use of the stationary phase minimizes sample preparation requirements; (2) parallel separations enhance sample throughput; (3) ease of post-chromatographic derivatization improves method selectivity and specificity; and (4) several screening protocols for different analytes can be carried out simultaneously.

Thin-layer chromatography remains one of the main methods for class fractionation and speciation of lipids [17,18] and is used increasingly to determine the botanical origin, potency, and flavor potential of plant materials (e.g. herbs and spices) [19–21]. In the pharmaceutical industry, it is used for the analysis of complex and dirty samples with poor detection characteristics and for stability and content uniformity testing [22–25]. It continues to be widely used for the standardization of plant materials used as traditional medicines [26]. Combined with digital autoradiography thin-layer chromatography is frequently selected as the method of choice to study the metabolism and fate of radiolabeled compounds in the body and environment [27,28]. In addition, thin-layer chromatography retains a historic link with the characterization of dyes and inks and the control of impurities in industrial chemicals.

In other cases, high-pressure liquid chromatography is the preferred technique, particularly if a large plate number is required for a separation, or the separation time is short compared with the sample preparation time. Separations by high-pressure liquid chromatography are easier to automate for unattended operation. Separations by size-exclusion and ion-exchange chromatography are more straightforward by high-pressure liquid chromatography. There are few suitable layers for the separation of biopolymers by thin-layer chromatography, and the use of thin-layer chromatography for these applications is minor compared with column chromatography and electrophoresis. For trace analysis, a wider range of selective detectors is available for high-pressure liquid chromatography compared with thin-layer chromatography.

3. Kinetic limitations

The common method of development in thin-layer chromatography employs capillary forces to transport the mobile phase through the layer. These weak forces arise from the decrease in free energy of the solvent as it enters the porous structure of the layer. For fine particle layers capillary forces are unable to generate sufficient flow to minimize the main sources of zone broadening [4,11,29,30]. In addition: (i) the mobile phase velocity varies as a function of time (and migration distance); and (ii) the mobile phase velocity is established by the system variables and is otherwise beyond experimental control. This results

in a slow and variable mobile-phase velocity through the layer with separation times that are longer than necessary. Separated zones are broader than they would be for a constant and optimum mobile phase velocity and the zone capacity limited by the useful range of mobile phase velocities. Typical separation systems afford a zone capacity of about 10–14, with few options to reach a higher value by optimizing the layer structure [1]. Multiple development provides a general approach to increase the zone capacity by taking advantage of a focusing mechanism that occurs each time the solvent front passes over the stationary zone on the layer. This affords perhaps a doubling of the zone capacity at the expense of longer separation times. Multiple development with an incremental increase in the development length and a decreasing solvent strength gradient is the basis of separations by automated multiple development [31]. There are no exact models for multiple development [32–34]. Results from phenomenological models indicate that further improvements over those already realized are unlikely for capillary flow systems, and there is no obvious solution to the significant increase in separation time. The magnitude and range of capillary flow velocities fundamentally limit separations by thin-layer chromatography. Faster separations with an increase in zone capacity require a higher mobile phase velocity than is possible for capillary flow as well as a velocity that is independent of the solvent front migration distance.

Forced flow separations overcome the principal deficiencies of capillary flow separations by establishing a constant and optimum mobile phase velocity. Forced flow separations require specially designed developing chambers in which migration of the mobile phase is driven by centrifugal force (rotational planar chromatography); by a constant volume reciprocating-piston pump (overpressured layer chromatography); or by an electric field (planar electrochromatography). Centrifugal methods are more popular for preparative-scale separations and are not discussed further [3,5,35,36]. Overpressured layer chromatography is better suited for analytical applications but can be used for preparative-scale separations as well. The construction and operation of the overpressured development chamber can be simplified for low-pressure separations by using a simple hydrostatic device as the mobile phase source [37]. As the challenges and limitations of overpressured layer chromatography and planar electrochromatography are different, it is convenient to treat them separately in the following sections.

3.1. Overpressured layer chromatography

Overpressure layer chromatography is an old technique in need of a breath of new life [3,38,39]. When first introduced its use was limited by the need for time-consuming plate preparation procedures, complications resulting from the disturbing zone, and awkward and unreliable instrumentation. The latter was probably the main cause of disenchantment with the technique, and although new instruments offer user-friendly features and are manufactured to higher standards, overpressured layer chromatography has not recovered from this false start. Few laboratories currently use forced flow based on the overpressure development chamber, and despite easy to identify advantages (see below), its long-term future is difficult to judge.

For forced flow separations in the overpressured development chamber, the layer is sealed on its open side by a flexible membrane under hydraulic pressure, and a pump is used to deliver the mobile phase to the layer. A constant mobile phase velocity independent of the solvent-front migration distance is obtained as long as the hydraulic pressure applied at the membrane maintains an adequate seal with the layer. This arrangement is not unlike column liquid chromatography except that the mobile phase initially encounters a dry layer. When a solvent is forced through a dry layer of porous particles sealed from the external atmosphere, the air displaced from the layer by the solvent usually results in the formation of a second front (beta front), moving behind the solvent–air front (alpha front), which is often wavy in character. The space between the alpha and beta fronts is referred to as the disturbing zone and consists of a mixture of solvent and gas bubbles. Sample components moving in the disturbing zone, or passed over by it, are often distorted and difficult to quantify by densitometry. In practice, the disturbing zone can be eliminated or minimized by predevelopment of the layer with a weak solvent in which the sample does not migrate [40]. The solvent

dislodges trapped air from the layer before starting the separation. Alternatively, a backpressure restrictor can be used to increase the local pressure and thus the solubility of the air in the mobile phase [41].

For forced flow separations, the plate height is independent of the solvent-front migration distance and achieves a minimum value for high-performance layers of about 22–25 μm over a narrow range of mobile phase velocities of 0.3–0.5 mm/s [1,3–5,30,42]. The plate height depends on the migration distance for capillary flow and has a minimum value of about 50 μm for a solvent-front migration distance of 4 cm. As expected, forced flow provides more compact zones and faster separations than capillary flow, and a significantly higher zone capacity (30–40 compared with 10–14) because of the higher intrinsic efficiency and the use of longer separation distances. For example, see Fig. 1. Other characteristic properties for layers with different particle sizes and a good column are summarized in Table 2 [4,5].

The variation of the reduced plate height with the reduced mobile phase velocity for forced flow separations on a high-performance layer and for a packed column for high-pressure liquid chromatography is illustrated in Fig. 2 [1,30]. The optimum reduced mobile phase velocity for the layer is shifted to a lower value compared with the column and the minimum in the reduced plate height (≈3.5) is higher than the column value (≈2.0–2.5). For higher reduced velocities, the reduced plate height for the layer increases significantly faster than for the column. Therefore, separations by forced flow are slower than for columns at optimum operating conditions, and separations at higher than optimum flow-rates considerably less efficient than columns. Evaluation of the coefficients for the Knox equation, Table 2, indicates that high-performance layers have a good packing structure but poor mass transfer properties [30,42]. A possible reason for the observed difference in properties is the result of the use of a binder to immobilize the layer. Measurement of pore volumes indicates that most of the binder is stored in the intraparticle volume, Table 2 [43]. The binder is thought to influence mass transfer by either restricting diffusion within the porous particles or by a multi-site adsorption mechanism with a range of association constants [42,43].

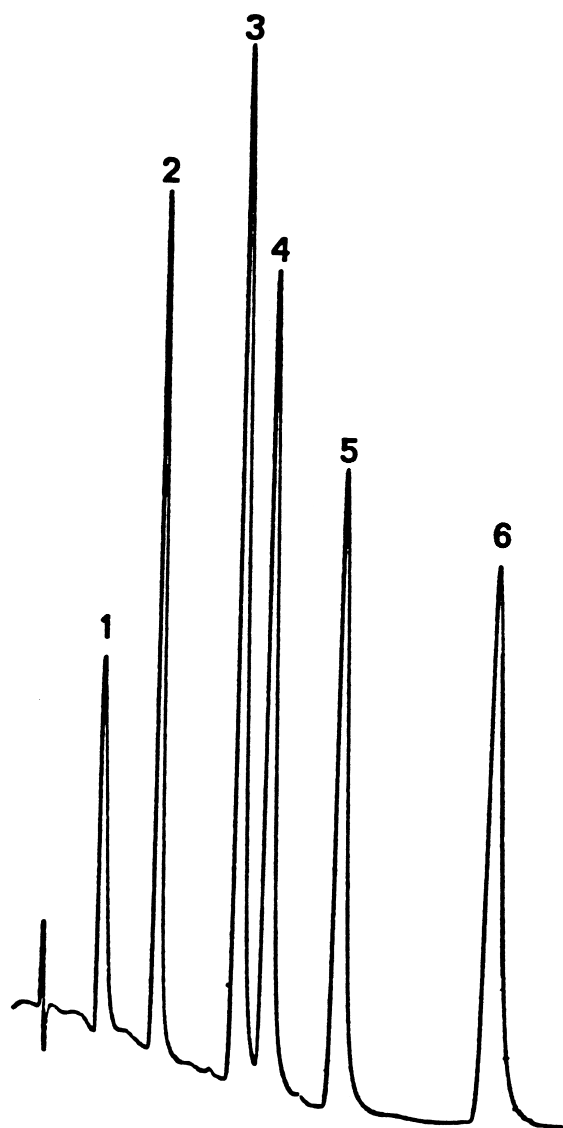

Fig. 1. Separation of a mixture of aromatic hydrocarbons by overpressured layer chromatography with on-line UV detection. Conditions: Merck Si-60 HPTLC plate, plate length 18 cm, mobile phase hexane at u=0.07 cm/s and inlet pressure 6.1 bar. Identification: 1, benzene; 2, naphthalene; 3, biphenyl; 4, phenanthrene; 5, fluoranthene; 6, triphenylene (from Ref. [4]. ©Elsevier).

Some expectations for forced flow separations are summarized in Table 3 [1,44]. For a development distance of 18 cm, a modest increase in performance (a maximum plate number of 8000) in a credible time of 9 min is achieved compared with typical results for capillary flow (<5000 theoretical plates in

Table 2
Characteristic properties of silica gel precoated layers and columns

Parameter	Layers		Column
	High performance	Conventional	High performance
Porosity			
Total	0.65–0.70	0.65–0.75	0.8–0.9
Interparticle	0.35–0.45	0.35–0.45	0.4–0.5
Intraparticle	0.28	0.28	0.4–0.5
Flow resistance parameter	875–1500	600–1200	500–1000
Apparent particle size (μm)	5–7	8–10	d_p
Minimum plate height (μm)	22–25	35–45	2–3 d_p
Optimum velocity (mm/s)	0.3–0.5	0.2–0.5	2
Minimum reduced plate height	3.5–4.5	3.5–4.5	1.5–3
Optimum reduced velocity	0.7–1.0	0.6–1.2	3–5
Knox equation coefficients			
Flow anisotropy (A)	0.4–0.8	1.7–2.8	0.5–1.0
Longitudinal diffusion (B)	1.2–1.6	1.2–2.0	1–4
Resistance to mass transfer (C)	1.4–2.4	0.70–0.85	0.05
Separation impedance	10,000–20,000	11,000–13,000	2000–9000

about 25–45 min). A significant increase in the plate number is available only by the use of longer layers at the expense of separation time. This can be achieved by the series coupling of several layers in a stack with a special tail-to-head vertical connection between layers [39,45]. The optimum mobile phase velocity is sufficiently low that the pressure drop remains modest compared with typical coupled column systems. Fast separations on high-performance layers result in low efficiency, and require high pressures if long development distances are used. This scenario is not very productive for layers. Reducing the particle size from an average of 5 to

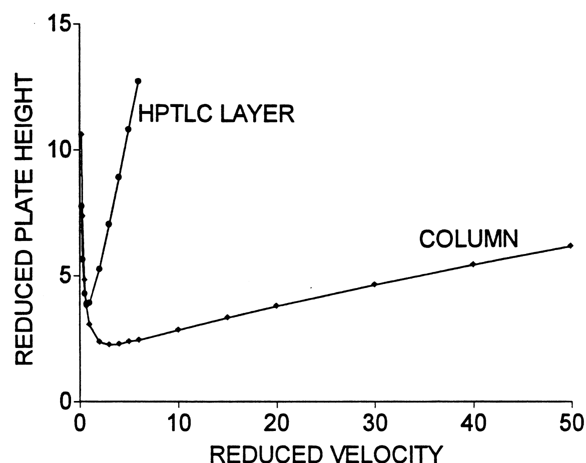

Fig. 2. Plot of the reduced plate height against the reduced mobile phase velocity for a high-performance layer by forced flow and an ideal column for high-pressure liquid chromatography (from Ref. [1]; ©Elsevier).

Table 3
Performance characteristics for forced flow thin-layer chromatography

Development time (min)	Pressure drop (atm)	N_{max}	Development length (cm)
HPTLC (optimum conditions $h=3.75$, $\nu=0.8$, $\phi=800$ and $d_p=6$ μm)			
4	2.1	3550	8
9	4.7	8000	18
25	12.9	22,200	50
50	25.8	44,400	100
HPTLC (fast development option $h=9$ and $\nu=5$)			
0.6	12.9	1480	8
1.4	29.1	3330	18
4.0	80.7	9250	50
8.0	161.0	18,500	100
HPTLC ($d_p=3$ μm; other parameters as in optimum conditions)			
2.0	16.5	7610	8
4.5	37.2	17,100	18
12.5	103	47,600	50
25.0	207	95,200	100

Assumptions: viscosity $=3.5\times10^{-4}$ N s/m^2 and solute diffusion coefficient $=2.5\times10^{-9}$ m^2/s (h, reduced plate height; ν, reduced mobile phase velocity; ϕ, flow resistance parameter; d_p, average particle size).

3 μm, however, provides an attractive range of efficiency values with a favorable separation time, but is more demanding in terms of operating pressure. These conditions are the most favorable for general use in overpressured layer chromatography, but require the design of new instruments capable of higher operating pressures, up to about 200 atm. Instruments available today are designed for operation at <50 atm. Significant improvements in performance could be achieved by instruments operating at about 100 atm with layers up to 50 cm long.

3.2. Planar electrochromatography

Electroosmosis provides a suitable alternative transport mechanism to pressure-driven flow in open-tubular and packed capillary liquid chromatography [5]. Electroosmotic flow in packed capillary columns is the basis of capillary electrochromatography. As well as providing a constant and optimum mobile phase velocity, the plug-like flow profile reduces transaxial contributions to band broadening. In addition, the mobile phase velocity is independent of column length and average particle size up to the limits established by double layer overlap or Joule heating. The general interest created by the rapid development of capillary electrochromatography as a useful separation method has trickled over to thin-layer chromatography, where in principle, electroosmotically-driven flow could provide an effective solution to the limitations of capillary flow.

The current status of electroosmotically-driven flow in thin-layer chromatography is probably more confusing than reassuring, although recent studies have brought some enlightenment to this technique. Early studies of electroosmotic flow in vertically mounted layers using solvents of low polarity as the mobile phase are now believed to be the result of thermal effects [46–48]. Enhanced flow is caused by forced evaporation of the mobile phase from a solvent-deficient region at the top of the layer. Because of drainage in vertically mounted layers, electrical resistance is highest at the top of the layer, and the increase in heat production drives the evaporation of solvent, pulling additional solvent through the layer. The most convincing proof of this mechanism is that in the absence of an electric field the results can be duplicated using a cartridge heater to heat a region of the top portion of the layer. True electroosmotic flow was demonstrated in horizontal developing chambers for both silica gel and chemically bonded layers. As would be expected from theory, solvents of low polarity provide limited or no electroosmotic flow [3,49,50]. Polar organic solvents and aqueous solutions provide reproducible and sustainable electroosmotic flow for conditions suitable for optimized separations [51–55]. However, virtually all studies so far describe the phenomena, no comprehensive studies of zone broadening have been undertaken, and no practical applications, beyond the separation of simple model mixtures demonstrated. In an open system like thin-layer chromatography, evaporation of mobile phase from the layer surface competes with electroosmotic flow along the layer. The voltage, pH and buffer concentration must be optimized to minimize either excessive flooding or drying of the layer to avoid degradation of the separation quality. These processes are probably better controlled by enclosing the layer and improving the thermostatting of the system. Since high pressures are not involved, mechanisms for enclosing the layer could be relatively simple compared to pressure-driven forced flow and new approaches (or older ones [44]) suggest that effective temperature control is possible [56,57]. Thinner layers may also help to contain temperature gradients in combination with adequate thermostatting [58].

Many questions remain unanswered: which solvents can be used; the need for prewetted layers and ions as current carriers; the effect of local heating on zone profiles; and the effect of binder chemistry on flow characteristics and mass transfer properties. Particle layers are generally immobilized by polymerization of ionic monomers and the affect that these binders have on the electroosmotic properties and electrical resistance of precoated layers is not understood. The most consistent results to date have been obtained with aqueous–organic solvent mixtures containing 1 m*M* buffer on reversed-phase layers with preadsorbed buffer ions, Fig. 3 [54,55]. Joule heating essentially limited solvent-front migration distances to about 4 cm. To make the method more attractive, longer development distances (≥10 cm) are required to obtain a significant increase in zone capacity compared with capillary flow. Kreibik

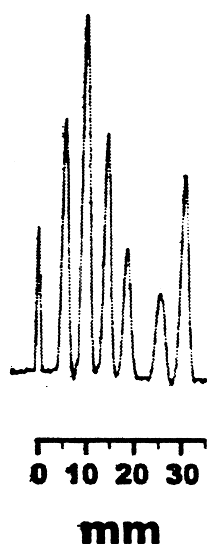

Fig. 3. Separation of a test mixture (4-cholesten-3-one, 17α-acetoxyprogesterone, 2′-acetonaphthone, benzanilide, 2-nitroaniline, 3,4-dimethoxybenzoic acid and 4-hydroxybenzoic acid) by planar electrochromatography. Separation conditions: Merck RP-18 F_{254}s layers with a mobile phase of 55% aqueous acetonitrile containing 25 m*M* sodium acetate buffer at pH 4.5 at 1000 V (from Ref. [54]; ©Elsevier).

et al. [59] have demonstrated in principle that external alternating electric fields can be used to drive solvents through a porous medium by dielectroosmotic flow. The usefulness of this mechanism for forced flow thin-layer chromatography, however, remains to be demonstrated.

3.3. Performance dilemma

The current dilemma in thin-layer chromatography is that there is little prospect of further improvements in separation performance so long as capillary flow is used as the mobile phase transport mechanism through the layer. Capillary flow thin-layer chromatography will never disappear completely. It will always have a role as a simple, transportable and near-instrument free technique to support rapid and simple mixture separations, such as monitoring organic reactions or combining column fractions from preparative chromatography. The advantages of overpressured layer chromatography are now clear, but there is a lack of confidence in the technology, prejudiced by first generation instruments and the limited visibility of more recent equipment in the Western Hemisphere. Even in this case, improvements in separation quality are possible with instruments capable of higher operating pressures. Electroosmotically-driven flow has caught some excitement from capillary electrochromatography but its implementation is not straightforward. It may be the way ahead because it is new, in principle simple, but practically infantile in its development. Before it can be seen as a competitive method to capillary or overpressured layer chromatography, the conditions and instrumentation for its application need to be convincingly demonstrated. Mobile phase compositions may be restricted to polar and aqueous solvents unsuitable for some, particularly normal-phase, separations. This may leave room for the co-development of forced flow. In either case, more research activity is required in both techniques if the introduction of these methods is not to be delayed and thin-layer chromatography further diminished because of inadequate separation performance compared with column chromatography.

4. Image analysis

Slit-scanning densitometry is the dominant method of recording thin-layer separations for interpretation and quantification [3,5,60]. This technology is now relatively mature, and although limited to absorption and fluorescence detection in the UV–visible range, has adequately served the needs of thin-layer chromatography for the last two decades. Evolution of slit-scanning densitometry is now largely progressive and major changes in operation and performance seem unlikely. A possible exception is the development of scanners employing a fiber optic bundle for illumination of sample zones and collection of reflected light (or fluorescence) in conjunction with a photodiode-array detector for simultaneous multiwavelength detection and spectral recording [61–64]. This approach simplifies data acquisition for some applications and affords the possibility of facile application of modern chemometric approaches for data analysis. This approach may improve the quality of available data from thin-layer separations, but does not overcome the principal limitations of slit-scanning densitometry. Significant improvements in

detection limits are also unlikely. Major improvements may be realized through image analysis, also known as video densitometry.

For video densitometry optical scanning takes place electronically, using a computer with video digitizer, light source, monochromators and appropriate optics to illuminate the plate and focus the image onto a charged-coupled device (CCD) video camera [3–5,65–67]. The main attractions of video densitometry for detection in thin-layer chromatography are fast and simultaneous data acquisition from the whole plate; a simple instrument design without moving parts; increase in sensitivity by using longer acquisition times; and compatibility with data analysis of two-dimensional chromatograms. There is increasing confidence that software for post-chromatographic data processing can be used to evaluate and minimize chromatographic and scanning errors resulting in improved data quality. The potential use of two-dimensional separations for thin-layer chromatography with image analysis techniques is discussed in Section 4.1. Video densitometry has proven popular in the development of field-portable instruments and as a replacement for photographic documentation of thin-layer separations. Modern instruments provide attractive options for searching and comparing sample images as well as integration of peak areas [3,68,69]. The ease of storing raw images for future applications and of pasting images into documents is responsible for a significant amount of the commercial success of video densitometers in current production. Office scanners have been shown to afford a simple, low-cost option for documentation of thin-layer separations with surprisingly good performance [70,71].

As things stand today, video densitometry cannot compete with slit-scanning densitometry in terms of sensitivity, resolution and available wavelength measuring range. A major difference in the two techniques, however, is that while slit-scanning densitometry is a stable technology, image analysis is rapidly evolving, driven by broad applicability to many analytical techniques, of which thin-layer chromatography is an obvious application but relatively minor component of the total potential market. Thus, one can be optimistic in thinking that thin-layer chromatography will benefit from developments in other areas of analytical sciences and from market conditions that reduce costs. Specific problems affecting thin-layer chromatography are inhomogeneous illumination of the layer during acquisition of the image, which is the main source of scanning error. Proper lighting can increase the image contrast and resolution, improving the overall performance of the system. At present, there is no system that affords uniform illumination of standard sized layers with the required spatial homogeneity for high resolution and high sensitivity measurements. Most low-cost instruments are designed for operation in the visible region or UV region when layers containing a fluorescence indicator excited at $\lambda = 254$ or 366 nm are used. In fact, one of the persistent problems in video scanning in general, remains the insensitivity of CCD cameras in the UV spectral region. Back-thinned CCD cameras provide much higher efficiency in the UV spectral range, but are currently very expensive. The possibility of spectral recording is a desirable feature for applications in thin-layer chromatography that is currently unsupported by video densitometry.

A fundamental limitation of both slit scanning and video densitometers is that the signal intensity in the reflectance mode is highly dependent on the vertical distribution of the sample within the layer. Image analysis is not as sensitive as slit-scanning densitometry to the in-depth concentration gradient, and although it would seem possible that this problem could be handled through software, no adequate solution has been proposed. The interaction of light with a particle layer is very complex, and it is probably the lack of a comprehensive model for this process, that has inhibited the solution to this problem.

4.1. Two-dimensional separations

Multidimensional separations employing two or more coupled orthogonal separation systems represent the preferred approach in chromatography to obtain a high peak capacity for the separation of complex mixtures [5]. In theory, two-dimensional separations are easily performed using planar separation systems [4,72,73]. Even capillary flow separations can be expected to afford a zone capacity of a few hundred rising to a few thousand for forced flow or automated multiple development, Table 4

Table 4
Zone capacity for different separation conditions in thin-layer chromatography

Separation mode	Dimension	Zone capacity
(i) Predictions from theory		
Capillary flow	1	<25
Forced flow	1	<80 (up to 150 depending on pressure limit)
Capillary flow	2	<400
Forced flow	2	Several thousand
(ii) Experimental observations		
Capillary flow	1	10–14
Forced flow	1	30–40
Capillary flow (AMD)	1	30–40
Capillary flow	2	≈100
(iii) Predictions based on (ii)		
Forced flow	2	≈1500
Capillary flow (AMD)	2	≈1500

[1,74,75]. It is likely that current theory overestimates the real separation potential of two-dimensional thin-layer chromatography, but even so, the more realistic estimates by experiment are still quite impressive. Also, the potential increase in zone capacity obtainable by using multiple development or forced flow to minimize zone broadening and the use of solvent strength gradients, as part of the optimization strategy, seem to have largely gone unrecognized [1,4]. One has to ask then, if the conditions for a high separation capacity are favorable for two-dimensional thin-layer chromatography and the experimental implementation seemingly relatively straightforward, why is the method so little used at present?

Some potential methods for generating two different retention mechanisms in orthogonal directions are summarized in Table 5 [4,76–80]. Using two solvent systems with complementary selectivity is the simplest approach but is often only partially successful. In most cases the two solvent systems differ only in their intensity for a given set of intermolecular interactions, and are not truly complementary. Such systems are responsible for the low success of two-dimensional separation systems to provide a significant increase in the separation potential apparent in many applications. Recent reports are more encouraging and recognize the importance of the orthogonal nature of the retention mechanisms if a high separation capacity is to be achieved. Bilayer plates with a smaller reversed-phase strip along one edge of the plate adjacent to a larger silica gel layer have provided the most popular approach for the implementation of two-dimensional separations with a high separation capacity. Chemically bonded layers can be used in the reversed-phase and normal-phase mode and allow the use of additives and buffers as a further means of adjusting

Table 5
Methods for generating two different retention mechanisms in orthogonal directions for thin-layer chromatography

- Develop in orthogonal directions with two solvent systems exhibiting different selectivity for the sample components
- Use a bilayer plate prepared from two sorbents with different selectivity for the sample. The sorbent layer for the first development is a narrow strip that abuts the much larger area used for the second development. Commercially available plates have silica gel and reversed-phase layers as adjacent zones
- Use a layer prepared from a mixture of two different sorbents and develop with different mobile phases such that the retention mechanism for the two, orthogonal, developments is governed by the properties of one of the sorbents in each direction
- After the first development the selectivity of the layer is changed by impregnation with a chemical reagent or immiscible solvent prior to the second development
- After the first development the properties of the sample are modified by chemical reaction or derivatization before the second development

selectivity. Aminopropylsiloxane-bonded layers can be used as an ion-exchange system with acidic mobile phases and as a reversed-phase or normal-phase system with a neutral or basic mobile phase. In several reports as many as 20 to 30 components were successfully separated by two-dimensional thin-layer chromatography indicating the potential of this approach for analyzing complex mixtures, even, as seems likely, the conditions used were not truly optimum [74,76–79].

The main problem preventing the general use of two-dimensional thin-layer separations is not the difficulty of obtaining the separations per se but the difficulty in analyzing the data. The acceptance of two-dimensional thin-layer chromatography for general analysis rests on providing a convenient method for in situ detection and quantification. Slit-scanning densitometers are designed for lane scanning and are not ideal for area scanning. Adaptation to scanning two-dimensional separations was demonstrated using normal scanning operations with small steps between scans or by zigzag scanning. Special software is required to map the layer surface and define zone locations and their optical density as three-dimensional plots or contour diagrams [4,81–83]. Slit scanning is slow using either small steps or zigzag scanning, and although these methods were described some time ago, they have not been used to any significant extent. Thus, it is not possible to provide any reliable comments on the quality of typical data produced by these methods.

The awaited breakthrough in general detection for two-dimensional planar separations is likely to come from video densitometry. Data acquisition is straightforward since the whole plate is imaged simultaneously, but a problem remains with quantification that has still to be addressed. In the absence of an accurate phenomenological model for the absorption (or fluorescence) process in densitometry calibration is required for conversion of intensity data to sample concentrations. Since only a single sample per plate is separated by two-dimensional thin-layer chromatography the normal methods of calibration used for lane scanning is not appropriate. The simplest solution is to separate samples and standards on different plates. The methods employed for integrating the absorbance of each zone, defining zone boundaries, and subtracting the background contribution from the layer become increasingly important if meaningful quantitative data is to be obtained. The possibility of quantitative analysis of two-dimensional separations with video densitometry is still to be demonstrated, but no doubt would result in a resurgence of interest in this approach.

5. Coupled column–layer separations

Multidimensional systems involving the coupling of two different separation techniques through a suitable interface provides a further approach to increasing the peak capacity over single column or layer techniques. Earlier reviews detail general approaches for column–layer coupling, which have advanced little since publication of the cited articles [1,4,84]. As well as increasing the separation capacity by column–layer coupling, it is hoped to access additional advantage by exploiting the specific attributes of layer separations, Table 6. Coupling gas chromatography to thin-layer chromatography (GC–TLC) is straightforward but has not been widely used since the late 1960s when several interfaces were described [84]. Typical problems for which these instruments were developed are solved by gas chromatography–mass spectrometry today, allowing only a weak case to be made for a likely resurgence of interest in GC–TLC. A suitable interface for coupling supercritical fluid chromatography to thin-

Table 6
Specific reasons for coupling column separation systems and layers

- The layer functions as a separation and storage device retaining information from the column and thin-layer separations in an immobilized format
- Sample components can be investigated free of time constraints, which is advantageous for: biomonitoring (see Section 8); for samples that require derivatization for detection; for sequential evaluation using different detection principles; and to preserve and transport the separation to different locations for evaluation
- To facilitate applications employing solid-phase spectroscopic identification techniques

layer chromatography (SFC–TLC) has been described [85]. Decompression of the supercritical fluid at a capillary orifice occurs with rapid cooling, favoring the deposition process without disturbing the conversion of the fluid to a gas. Efficient transfer of sample to the layer requires solvent addition, to minimize sample loss through formation of high-speed particles. Wet particle deposition being more efficient than dry particle deposition. The coupling of capillary electrophoresis to thin-layer chromatography (CE–TLC) was described recently, but no real applications were demonstrated [86]. Current and past interest is largely limited to the coupling of column liquid chromatography to thin-layer chromatography (LC–TLC), which has reached a reasonable level of maturity supported by commercially available interfaces.

5.1. Liquid chromatography–thin-layer chromatography (LC–TLC)

The most general interface for coupling column liquid chromatography to thin-layer chromatography (LC–TLC) is based on different modifications to the spray-jet applicator [1,4,87–91]. At flow-rates typical for packed capillary columns (5–100 μl/min) the total mobile phase can be applied to the layer. A splitter in the transfer line to the spray-jet applicator is required to accommodate higher flow-rates from wider bore columns. The column eluent is nebulized by mixing with (heated) nitrogen gas and sprayed as an aerosol onto the layer. The spray head is moved horizontally on one line within a defined bandwidth or, better, is made to deposit the spray over a defined rectangular area (e.g. 8×6 mm) to promote effective solvent evaporation. In the latter case, the zone is focused by a short development with a strong solvent before separation.

In the profiling mode, the whole column chromatogram is divided into volume fractions sequentially transferred to the layer and deposited as a series of bands that are subsequently developed in parallel. Each track (band) is scanned individually revealing an immense amount of information about the sample composition. In the target compound mode, fractions identified by the column detector, or from elution windows established by marker compounds, are transferred to the layer and stored there. When all the available space is occupied by column fractions from a single or several samples, the layer is developed and evaluated. The main limitations of the spray-jet interface are its restricted flow capability and inability to handle non-volatile ionic additives, such as buffers and ion-pair reagents. These problems can be overcome by combining an automated solid-phase extraction module with the spray-jet applicator to concentrate column fractions and exchange the solvent for layer deposition [92].

Contemporary interest in LC–TLC remains weak. The main problems are more on the detection and data handling side than separations. It is simpler to obtain mass spectral information from the solution phase using liquid chromatography–mass spectrometry (LC–MS) than to either quantify or identify separated bands by thin-layer chromatography–mass spectrometry (TLC–MS), discussed in Section 6. Solid-phase spectroscopic techniques, such as surface enhanced Raman spectroscopy (SERS), photoacoustic spectroscopy and fluorescence line-narrowing spectroscopy (FLNS), provide a rich source of structural information for compound identification. Most analysts, however, are unfamiliar with these techniques and so are unlikely to seek out this information to characterize unidentified compounds [93–95].

A large amount of information is produced by lane scanning densitometry of LC–TLC separations, and is presented in a form that is difficult to analyze. Individual sample components may be present in more than one lane and quantification is often inconvenient or impossible due to the need for calibration for optical detection. Video densitometry (Section 4) could prove more useful for data management and comparison. A convenient and routine approach to in situ mass spectrometry (Section 6) could impact considerably on the wider use of LC–TLC. Complex mixtures tend to contain many unknown substances and require a convenient method for identification. Increasing interest in bioactivity detection (Section 8) suggests a possible catalyst for growth in this technique. In the author's opinion, LC or (SFC)–TLC, will not become a widely used separation tool in the absence of improvements in both the quality and ease of use of data obtained from layer separations. It will grow as a technique only when the conditions are right, and until then

interest will remain limited, very much as it is at present.

6. Mass spectrometry

The combination of chromatographic separations with mass spectrometric detection is considered an indispensable tool for problem solving in analytical chemistry and increasingly for routine analytical methods [5]. Mass spectrometric detection brings an added level of information, complementary to the chromatographic process, that improves the certainty of identification and the specificity of detection. Mass spectral information can generally be obtained from sample sizes typical of common analytical methods. Robust and affordable coupled instruments for GC–MS and LC–MS are available in many laboratories and CE–MS, if not as widely available, is adequately supported by the commercial sector. By comparison, TLC–MS is mainly a research tool available to a small number of research groups, which have mainly constructed their own interfaces. The evolution of TLC–MS has been slow compared with LC–MS. In practice, mass spectra of samples separated by TLC are generally obtained by slow and labor-intensive manual methods (e.g. solvent extraction of excised zones with conventional liquid introduction into the mass spectrometer). The challenge is to develop an automated system for in situ acquisition of mass spectral data directly from layers with retention of the spatial integrity of the chromatographic separation. This is certainly not a simple problem, but is a problem of some importance, since it restricts the range of applications that TLC is considered suitable and diminishes interest in those TLC techniques proposed for the analysis of complex mixtures.

The problems of interfacing thin-layer chromatography to mass spectrometry (TLC–MS) are largely different to those experienced in LC–MS and other column separation techniques [3,94,96–99]. At the completion of the separation the chromatogram is fixed in time and space with the major portion of the mobile phase eliminated by evaporation. The thin-layer plate can be considered as a storage device effectively decoupling the separation requirements from those of measuring the mass spectra. The immobilized separation is also generally compatible with the vacuum requirements of the mass spectrometer. On the other hand, the sample is embedded within the stationary phase layer from which it must be extracted into the gas phase and ionized for mass analysis. It is not surprising, therefore, that the principal methods of instrumental TLC–MS are based on surface desorption and ionization techniques using fast atom bombardment [97,99–101], laser desorption and ionization [102–104], and MALDI [97,105–109]. Commercial interfaces provide rather limited automation capabilities. Only in the literature are systems providing automated plate scanning, chromatogram recording and mass spectrum acquisition under software control for single isolated tracks, and less commonly with full plate scanning capability, described. As an alternative to these general approaches, probes for direct solvent extraction of individual separated zones followed by electrospray ionization of the extracted sample have been described [110,111].

To improve the sensitivity of the FAB method, a matrix solution or phase transition matrix is applied to the layer and spectral acquisition and time averaging of the ion signal employed. Because ions are removed from the surface only, the ionization efficiency will be low unless a mechanism is provided to extract and cycle the major portion of the sample to the surface in a continuous manner. Impregnating the chromatogram with a viscous liquid or low melting point solid (phase transition matrix) fulfills this role without destroying the integrity of the chromatogram through zone broadening. For reliable mass spectra in the scan mode, sample amounts in the tens of nanograms to microgram range are required. Since FAB ionization produces largely molecular ion adducts with limited fragmentation, tandem mass spectrometry and collision-induced dissociation are used for identification purposes [99–101].

Methods relying on MALDI require application of an extraction solvent to the layer to move sample to the layer surface followed by co-crystallization with a MALDI matrix material. TLC–MALDI direct coupling methodologies use one of four methods of layer treatment. A MALDI matrix solution is deposited directly on the layer, or better still, applied to the layer by electrospray [107] and the solvent

evaporated with crystallization of the matrix. The main problem with this approach is loss of separation integrity due to zone broadening caused by convection driven by matrix crystallization. The pressing method attempts to reduce sample spreading by separating the extraction and crystallization steps [105]. A MALDI matrix layer is formed on a smooth inert substrate, separate from the TLC plate, and is transferred to the surface of the separation layer by pressing the matrix layer and TLC plate face-to-face in the presence of a sprayed-on extraction solvent. The spatial resolution and detection limits are largely determined by the selection of the extraction solvent, the extraction time, the pressure and time used for the pressing step, and the thickness of the stationary phase. Even for optimum conditions the sample recovery remains low (<22%) because of the poor extraction efficiency of the sample from the sorbent pores as well as some lateral broadening of sample zones. The extraction efficiency is virtually complete using a hybrid TLC–MALDI plate, in which two juxtaposed layers, a TLC layer and a MALDI matrix layer, are formed on a common support. The separation is performed in one direction on the TLC layer, the mobile phase evaporated and the MALDI matrix applied to the MALDI zone, followed by elution of the sample in the direction of the MALDI matrix layer [106]. The MALDI matrix layer is used to acquire the mass spectra. The most recent approach uses a suspension of graphite particles in ethylene glycol and ethanol sprayed onto the layer as an energy-transfer matrix [108,109]. This approach improves the sensitivity and reduces the contribution of matrix ions to the mass spectral background. Optimization of all four methods is critical to their success with good quality mass spectra consisting of mainly molecular ion adducts with a few fragmentation ions obtained from nanogram amounts of sample.

The capabilities of LC–MS can be used to assess the level of satisfaction for TLC–MS, given that user desires are similar for both techniques. It is quite obvious that TLC–MS falls well below the satisfaction level of LC–MS. TLC–MS is generally used for obtaining spectra only and is not considered reliable for quantitative analysis. The possibility of chromatogram reconstruction from stored ion intensities or base peak ion intensities, the use of mass chromatograms for target compound location and automated identification by library searching is very limited in TLC–MS. A major advantage of liquid chromatography is the active transport mechanism of the mobile phase that delivers the whole sample to the mass spectrometer in a time defined by the peak widths. In thin-layer chromatography the sample is transferred to the surface of the layer slowly and incompletely by the extraction procedures in common use. This limits the possibility of mass imaging of the whole layer, or selected ion imaging to identify target compounds, and the use of ion intensity data for quantification. It is also necessary for TLC–MS to provide data in an automated fashion and in near real time if it is to become accepted alongside LC–MS. At present progress in this core technology is slow, primarily because it has never received the attention and financial investment that underpinned the evolution of LC–MS, itself a difficult problem.

7. Biomonitoring

Biomonitoring implies the use of a detection mechanism based on a biological response compared with conventional detection techniques that are based on a physical interaction or chemical reaction. Biomonitoring is rarely used to replace conventional detection techniques. Rather, it is considered a complementary tool that provides information relevant to the detection of compounds with a defined biological effect, such as toxicity, mutagenicity, growth inhibition of cellular components, enzyme inhibition, etc. For complex mixtures, bioassays are used to direct the analytical protocol for the isolation and identification of compounds with specific properties as an alternative to target compound analysis, where a conventional detector is used to evaluate the presence of a known compound in a complex mixture. In target compound analysis the analyst has to know in advance what compounds to look for and methods are usually limited to the analysis of a small number of compounds, often with similar properties. There is no possible assessment of whether the isolated compounds represent a large or small fraction of the biological effect for the sample. For bioassay-directed methods, no presumptions of com-

pound identity are required and samples are evaluated based on their biological activity resulting from the presence of known or unknown substances.

Thin-layer chromatography is well suited to biological detection since the separation is immobilized before detection and the open nature of the layer allows access to the sample. The use of multiple reaction steps including slow reaction procedures, such as incubation of bacterial cultures, is simplified, and the simultaneous separation of samples in parallel provides high sample throughput for screening. Column chromatographic methods generally provide higher resolution than thin-layer chromatography for complex mixtures but are less attractive for biomonitoring because many individual fractions have to be collected and then processed prior to the bioassay. The fractionation of samples by thin-layer chromatography and favorable coupling with biomonitoring eliminates the time-consuming preparation of fractions for biological testing and lowers the cost for sample evaluation. Normal-phase separations, commonly employed in thin-layer chromatographic methods, are in many cases advantageous for the identification of polar bioactive compounds that are poorly retained in reversed-phase liquid chromatography. Many reversed-phase column separations employ mobile phase solvents and additives that are themselves either active in general bioassays or incompatible with the bioassay, requiring extensive and complex sample work-up, with a negative effect on sample throughput. In addition, thin-layer chromatography facilitates the convenient evaluation of larger sample sizes than column chromatography by applying samples to the layer as bands rather than as spots.

The combination of bioassay-directed detection and separation by thin-layer chromatography is increasingly being recognized as a powerful alternative to conventional target compound analytical methods. In many applications the biological properties of a sample are more relevant than the identification of a limited number of sample components. Some typical examples include: toxicity-directed wastewater analysis [112–115]; rapid screening and isolation of bioactive compounds from medicinal plants [3,116–118]; detection of antimicrobial compounds in plants, feeds and foods [118–125]; isolation of radical scavengers and antioxidants in foods [116]; and the screening of combinatorial libraries for candidate drugs [126]. The isolation of novel biologically active compounds from plant and animal sources requires a dereplication step for the identification of known compounds to avoid their time-consuming isolation. Thin-layer chromatography with biomonitoring is the most efficient and least expensive method for activity-guided isolation of natural products [116].

Common methods of bioassay for thin-layer separations are based on (usually) the inhibition of growth or activity of a test organism, such as mould spores, yeast cells, bacteria, cell organelles (e.g. chloroplasts) or enzymes. Enzyme inhibition methods often provide the simplest approach. For detection the thin-layer plate is simply dipped or sprayed sequentially with a solution of the enzyme, substrate and dye (if required) to determine inhibition by color differentiation [115,117]. Ellman's method for cholinesterase inhibitors, for example, yields white zones on a yellow background with detection limits of 0.01 to 0.2 μg for inhibitor substances. Biologically active substances can be detected with good sensitivity by their inhibition of the luminescence of biological systems. The choice of the luminescent organism determines the specificity of the method. The bioluminescence of *Vibrio fischeri* is closely coupled to the metabolic status of the organism and has been developed into a widely used indicator of toxicity [114,116]. After separation the dried plate is briefly immersed in a suspension containing *Vibrio fischeri*, excess suspension is removed from the plates, and after a short incubation period, the bioluminescence is recorded with a video densitometer or by photography [112,114,116,124]. Toxic substances are revealed as dark zones resulting from reduced bioluminescence. Detection limits for toxic compounds are in the picogram range.

Antibacterial compounds can be detected by one of three methods: (i) agar diffusion (contact bioautography), (ii) agar overlay (immersion method), and (iii) direct methods. The agar-diffusion method is the oldest approach. Antibacterial compounds are detected by contacting the layer face-to-face with an inoculated agar plate. The separated zones are transferred to the agar gel by diffusion and inhibition zones visualized by use of suitable stains and (mainly) dehydrogenase-activity-detecting re-

agents [3,125,126]. In the immersion method a warm agar solution of the bacteria is poured over the layer and allowed to set. For Gram-negative bacteria an agar solution containing the red-colored bacterium *Serracia marcescens* can be used [126]. The red-colored gel is incubated overnight at room temperature and inhibition zones determined as white or pale yellow areas on a red background. For Gram-positive bacteria an agar gel containing the bacterium *Bacillus subtilis* is widely used. The colorless gel is incubated overnight at room temperature. After spraying with methylene blue, inhibitors are identified as white zones on a blue background. In direct bioassay methods, a suspension of a microorganism in a suitable broth is applied to the thin-layer plate [3,124]. The bacteria are allowed to grow by incubation in a humid atmosphere. Inhibitory zones are generally visualized based on the capacity of dehydrogenases of living microorganisms to convert tetrazolium salts into the corresponding intensely colored formazin. Antibacterial compounds on the thin-layer plate kill the microorganisms and appear as clear spots against a colored background.

The spray reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) is widely used for the identification of radical scavengers and antioxidants [116]. DPPH is a stable radical with a purple color that turns yellow after reduction. The assay consists of spraying the TLC plates with a 0.2% (w/v) DPPH solution in methanol. Plates are examined after 30 min. Active compounds appear as yellow spots against a purple background. β-Carotene is also used to detect antioxidants. The layer is sprayed with a solution of β-carotene, dried and exposed to 254 nm UV light. The β-carotene is bleached except in places where antioxidants prevent its degradation. Active compounds appear as orange zones on a pale background.

8. Biopartitioning

Several biopartitioning processes are used as an indicator of chemical properties in drug discovery, toxicity assessment, environmental fate, etc. Experiments in biological systems, however, can be difficult for technical and ethical reasons, as well as costly to perform. As a result, considerable interest exists in the development of surrogate chromatographic models for the timely and economic prediction of the outcome of biological processes [127–129]. Chromatographic methods are suitable for estimating biological properties as they are fast, require little material (which does not have to be pure), are relatively easy to automate and have favorable cost factors. Thin-layer chromatography has not been used to any significant extent for estimating biopartitioning properties, but has considerable potential for this application. The main obstacle has been the identification of suitable chromatographic models from the large number of possible thin-layer chromatography systems. This can now be tackled in a systematic way [127–129].

Abraham has described an approach for characterizing the properties of biopartitioning processes that is robust with respect to structure diversity [127]. The free energy property characteristic of the biological process is considered to result from contributions of cavity formation and intermolecular interactions in an aqueous phase and biological system according to the general equation:

$$\mathrm{BP} = c + vV + eE + sS + aA + bB \quad (1)$$

where BP is a free-energy related property of the biological system. The model equation is made up of product terms representing solute properties (descriptors) and the complementary properties of the biological system (system constants). The solute descriptors are McGowan's characteristic volume V, the excess molar refraction E, the solute's dipolarity/polarizability S, and the solute's effective hydrogen-bond acidity and hydrogen-bond basicity, A and B, respectively. The system constants are characteristic of the biological process. Each system constant (v, e, s, a, b) represents the difference in a defined property in the aqueous phase and the biological system. The e constant is determined by the contribution from electron lone pair interactions, the s constant by dipole–dipole and dipole-induced dipole interactions and the a and b system constants by hydrogen-bond basicity and acidity, respectively. The v constant is a measure of the dispersion interactions that fail to cancel when the solute is transferred from one phase to the other together with contributions from the difference in the ease of

cavity formation in each phase. Eq. (1) has been used to characterize a number of biopartitioning processes with selected results summarized in Table 7 [130–138]. A chromatographic system will emulate a biological process if the system constants for both processes are (nearly) identical. It will correlate with a biological process if the ratios of the system constants for both models are (nearly) identical. It is unlikely that there are more than a few possible emulation models but the requirements for a correlation model, usually of the type:

$$BP = mR_M + p \quad (2)$$

where m and p are constants and R_M the free energy related property of the thin-layer chromatographic system ($R_M = \log\ [1 - R_F]/R_F$), are more hopeful. This approach was successful for identifying chromatographic systems with similar separation properties as a component of computer-assisted method development [2,138–140].

The starting point for identifying potential surrogate thin-layer chromatographic models for the biopartitioning processes in Table 7 is to construct a database of thin-layer chromatographic system constant ratios that can be searched against the system constant ratios in Table 7. A suitable database for reversed-phase thin-layer chromatographic systems was compiled from literature sources, Table 8 [2,141–143]. The database was searched using two general filters to determine similarity. All thin-layer separation systems that differ in at least one system constant ratio by ≥0.2 from the equivalent ratio for the biopartitioning system were rejected. Secondly, if the sum of all differences between system constant ratios exceeded 0.5 the chromatographic system was considered to lack the required similarity in properties. Both filters were applied sequentially to identify surrogate TLC models for the biopartitioning systems. The advantage of this approach over conventional methods is that it removes the empirical nature from the selection process. Since there are a vast number of chromatographic systems that do not provide useful models for the biopartitioning systems, the probability of identifying suitable models by trial and error is remote.

No useful models were identified for intestinal adsorption, blood–brain distribution and non-specific toxicity to the golden orfe and *Vibrio fischeri* (microtox test). The fundamental absorption properties of these biological systems have a different character to the thin-layer separation systems. Useful correlation models were identified for the other biopartition-

Table 7
System constants for biopartitioning processes

Biological process	Dependent variable (BP in Eq. (1))	System constant ratios					Ref.
		v	e/v	s/v	a/v	b/v	
Intestinal absorption	%Abs	10.6	0.28	0.39	−2.05	−1.99	[130]
Blood–brain distribution	log BB	0.93	0.496	−0.926	−0.605	−0.78	[131,132]
Non-specific toxicity							
Fathead minnow	$-\log LC_{50}$	3.39	0.071	0	0.118	−1.077	[133]
Guppy	$-\log LC_{50}$	3.30	0.180	0	0.108	−0.946	[133]
Golden Orfe	$-\log LC_{50}$	2.80	0.50	0	0.364	−0.775	[133]
Tadpole	$-\log EC_{50}$	3.14	0.243	−0.219	0	−0.746	[134]
Vibrio fischeri (Microtox test)	$-\log PT_5$	3.73	0.290	0	0	−0.480	[135]
Tetrahymena pyriformis (Tetratox test)	$-\log IGC_{50}$	2.88	0.220	0	0	−0.872	[135]
Soil–water	$\log K_{OC}$	2.09	0.354	0	−0.148	−1.086	[136]
Plant cuticular matrix–water	$\log K_{MXw}$	3.91	0.153	−0.106	−0.130	−1.048	[137]

%Abs, percent absorption; BB is blood–brain partition coefficient expressed as [concentration of solute in the brain]/[concentration of solute in the blood]; LC_{50} is the lethal concentration of solute for 50% kill in a fixed exposure time; EC_{50} is the solute concentration required for 50% effect in a fixed exposure time; PT_5 is the concentration of solute (m*M*) causing 50% diminution of bioluminescence in 5 min; IGC_{50} is the concentration of solute causing 50% inhibition of cell growth in 40 h; K_{OC} is the soil–water distribution constant normalized to the percent carbon soil organic matter (Freundlich sorption coefficient/fraction of soil organic carbon); K_{MXw} is the water–plant cuticle matrix partition coefficient for the tomato fruit (*Lycopersicon esculentum*).

Table 8
Reversed-phase thin-layer chromatographic systems in the database of system constants

Layers (Merck HPTLC)	Solvent system	Ref.
RP-18 WF 254s	Methanol (0–90% v/v)	[142]
	2-Propanol (0–70% v/v)	[142]
	2,2,2-Trifluoroethanol (0–50% v/v)	[142]
	Acetone (0–90% v/v)	[142]
	N,*N*-Dimethylformamide (0–60% v/v)	[142]
	Pyridine (0–50% v/v)	[2]
	Acetonitrile (0–80% v/v)	[142]
CN F 254s	Methanol (0–90% v/v)	[141]
	2-Propanol (0–80% v/v)	[141]
	2,2,2-Trifluoroethanol (0–80% v/v)	[141]
	Acetone (0–80% v/v)	[141]
	N,*N*-Dimethylformamide (0–80% v/v)	[141]
	Pyridine (0–50% v/v)	[2]
	Acetonitrile (0–80% v/v)	[141]
	Methanol + acetonitrile ternary mixtures	[143]
DIOL F 254s	Methanol (0–50% v/v)	[2]
	Acetone (0–60% v/v)	[2]

ing processes, Table 9. The list length was restricted to the 10 closest fits for any biopartitioning process meeting the requirements established by the prefilter. Several TLC systems afford suitable models for non-specific toxicity with the fathead minnow, guppy, tadpole and *Tetrahymena pyriformis* (tetratox test) as the test organism. Suitable models are also identified for the soil–water distribution constant and the plant cuticle matrix–water distribution constant. The above search indicates that thin-layer chromatography exhibits a good potential as a source of surrogate models for estimating biopartitioning properties.

9. Conclusions

In this report, core technologies capable of affecting the practice and future of thin-layer chromatography are identified. The pace of development, however, will depend on the number of adequately resourced research groups committed to advancing these core technologies. Over the last decade, this has been a significant obstacle, as opportunities in other separation fields have appeared more attractive than those presented by thin-layer chromatography. Optimization of conditions for mobile phase transport should be an early priority. Full-working directions and manufacturing support should be established for either overpressured layer chromatography or electroosmotically-driven flow as the method of choice for mobile phase transport. It is unlikely that techniques with a high separation potential, such as two-dimensional and coupled column–layer systems, will gain in popularity in the absence of adequate methods for data analysis and identification of separated substances. Video densitometry has a significant role to play in this regard for both detection and on account of the flexible approaches afforded for data analysis. Mass spectrometry is the preferred technique for identification of separated substances, but there are no real clues as to how to develop robust interfaces for scanning and recording in situ mass spectra. More so if the full complement of identification and quantification tools available to column liquid chromatography is taken as the desirable goal for thin-layer chromatography. Bioactivity detection is easily interfaced with thin-layer chromatography and has the potential to become a powerful selective detection principle for the analysis of complex mixtures with minimal sample preparation. Similarly, thin-layer chromatography has good potential as a source of surrogate models for estimating biopartitioning properties in support of combinatorial chemistry and environmental fate and health effects studies. These last two applications will grow in importance with sufficient interest, and are not limited by any specific obstacle.

Table 9
Potential surrogate thin-layer chromatographic models for biopartitioning processes

Biopartitioning process	Difference in system constant ratios (Δ)					TLC system
	e/v	s/v	a/v	b/v	ΣΔ	
Fathead minnow	0.07	0	0.01	0.08	0.16	30% Pyridine–water on CN
	0.07	0	0.12	0.05	0.24	80% Acetonitrile–water on CN
	0.07	0	0.18	0.05	0.30	30% Acetonitrile–water on RP-18
	0.17	0	0.12	0.06	0.35	20% Acetone–water on CN
	0.10	0	0.12	0.13	0.35	20% 2-Propanol–water on CN
	0.07	0	0.12	0.17	0.36	40% 2-Propanol–water on CN
	0.15	0	0.09	0.12	0.36	20% Pyridine–water on CN
	0.14	0	0.12	0.11	0.37	20% Acetonitrile–water on CN
	0.10	0.06	0.18	0.05	0.39	40% Dimethylformamide–water on RP-18
	0.07	0	0.18	0.15	0.40	50% Pyridine–water on CN
Guppy	0.01	0	0.11	0.01	0.13	20% 2-Propanol–water on CN
	0.04	0	0.10	0.01	0.15	15% Pyridine–water on CN
	0.03	0	0.11	0.02	0.16	20% Acetonitrile–water on CN
	0.06	0	0.11	0.03	0.20	30% Methanol–water on CN
	0.07	0	0.11	0.03	0.21	40% Methanol–water on CN
	0.06	0	0.11	0.08	0.25	10% Acetone–water on CN
	0.01	0.06	0.11	0.08	0.26	40% Dimethylformamide–water on RP-18
	0.18	0	0.11	0.04	0.33	30% 2-Propanol–water on CN
	0.18	0	0.11	0.04	0.33	40% 2-Propanol–water on CN
	0.18	0	0.11	0.08	0.37	80% Acetonitrile–water on CN
Tadpole	0.02	0.04	0	0.09	0.15	60% Dimethylformamide–water on RP-18
	0.04	0.11	0	0.06	0.21	50% Dimethylformamide–water on RP-18
	0	0.08	0.14	0.01	0.23	10% Dimethylformamide–water on RP-18
	0.06	0.11	0.18	0	0.35	20% Acetonitrile–water on RP-18
	0.03	0.11	0.15	0.12	0.41	20% Dimethylformamide–water on RP-18
Tetrahymena pyriformis	0.02	0	0	0.03	0.05	10% Acetonitrile–water on CN
	0.05	0	0	0	0.05	10% 2-Propanol–water on CN
	0.03	0	0	0.04	0.07	40% Methanol–water on CN
	0.02	0	0	0.05	0.07	30% Methanol–water on CN
	0.06	0	0	0.03	0.09	5% Acetonitrile–water on CN
	0.01	0	0	0.09	0.10	20% Acetonitrile–water on CN
	0.09	0	0	0.03	0.12	10% Dimethylformamide–water on CN
	0.07	0	0	0.05	0.12	20% Methanol–water on CN
	0.05	0	0	0.08	0.13	20% 2-Propanol–water on CN
	0.05	0.06	0	0.16	0.27	40% Dimethylformamide–water on RP-18
Soil–water	0.06	0	0.15	0.03	0.24	5% Pyridine–water on CN
	0.19	0	0.06	0.14	0.39	50% Methanol–water on CN
	0.14	0	0.15	0.12	0.41	20% Acetonitrile–water on CN
	0.11	0	0.15	0.17	0.43	30% Methanol–water on CN
	0.18	0.07	0.15	0.06	0.46	40% Dimethylformamide–water on RP-18
	0.14	0.08	0.07	0.19	0.48	30% Acetonitrile–water on RP-18
	0.18	0.14	0.02	0.16	0.50	30% Dimethylformamide–water on RP-18

Table 9. Continued

Biopartitioning process	Difference in system constant ratios (Δ)					TLC system
	e/v	s/v	a/v	b/v	ΣΔ	
Plant cuticle matrix–water	0.02	0.05	0.13	0.02	0.22	40% Dimethylformamide–water on RP-18
	0.01	0.11	0.07	0.10	0.29	50% Methanol–water on CN
	0.02	0.11	0.13	0.10	0.36	20% 2-Propanol–water on CN
	0.09	0.11	0.13	0.03	0.36	10% Acetone–water on CN
	0.15	0.11	0.03	0.09	0.38	30% Dimethylformamide–water on CN
	0.15	0.11	0.09	0.04	0.39	40% Dimethylformamide–water on CN
	0.06	0.11	0.13	0.09	0.39	20% Acetonitrile–water on CN
	0.14	0.11	0.13	0.01	0.39	5% Pyridine–water on CN
	0.15	0.11	0.07	0.08	0.41	30% Acetone–water on RP-18
	0.15	0.11	0.15	0.03	0.44	40% Acetonitrile–water on CN

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