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Analytical Survey

Contemporary developments in thin-layer chromatography

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Abstract: Qualitative and quantitative applications of thin-layer chromatography (TLC) in the biomedical and pharmaceutical fields are briefly surveyed. Some recent developments, including high-performance TLC (HPTLC) and reversed-phase TLC (RPTLC), together with techniques such as multiple development and continuous development, are considered. Instrumentation for HPTLC, and new developments in the detection and spectroscopic characterization of substances on TLC plates, are briefly described.

Keywords: *Thin-layer chromatography; high-performance TLC; reversed-phase TLC; TLC–mass spectrometry; programmed multiple development in TLC; ion-pair TLC; radial TLC; overpressurized TLC.*

General Introduction

Although thin-layer chromatography (TLC) was first described over 40 years ago, it only came into general use following the pioneering work of Stahl [1]. The technique has since proved so useful that it is now employed in almost every area of biomedical and pharmaceutical research. Advances in other areas, particularly high-performance liquid chromatography, have resulted in some changes in the pattern of use of TLC, but there is no evidence of its relegation to the minor role now occupied by paper chromatography. Indeed it has been claimed that nearly 30% of all chromatographic publications have been devoted to TLC [2], and Sherma and Fried recently identified some 2000 publications in this area over the period 1981–1983 [3].

There are many reasons for the continued popularity of TLC. These include simplicity, speed, robustness and large sample capacity, combined with modest demands on equipment and resources. As a method for qualitative analysis TLC is probably unsurpassed, and the advent of reliable and sensitive TLC scanners has extended

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applications of the technique to quantitative analysis with detection limits at the ng, or even pg, level for suitable compounds. Whilst the power of HPLC for quantitative work is unquestioned, there are certain circumstances where TLC may have significant advantages. For instance, sample preparation for TLC is often less complex than for HPLC, and samples that would destroy an expensive HPLC column after a few injections can often be analysed without difficulty. Further, there are fewer restrictions on the types of solvent that can be used in TLC. Thus solvents that cannot be used for HPLC because of their high UV absorbance (e.g. toluene, acetone, ethylacetate) are suitable for TLC, where chromatography and detection are distinct processes.

The versatility of TLC can be extended by employing a vast range of post-chromatographic spray reagents to enhance the specificity and sensitivity of detection. This is analogous to the use of post-column reactions in HPLC; however, in the latter case the reagent must be compatible with the effluent from the HPLC column, a limitation which does not apply to TLC.

Since its discovery TLC has evolved into an important analytical tool. In this brief survey the current applications of the technique in the biomedical and pharmaceutical fields will be considered, and recent developments in techniques, equipment and the TLC plates themselves described.

Biomedical and Pharmaceutical Applications of Thin-Layer Chromatography

The general description "biomedical and pharmaceutical" analysis covers a broad spectrum of applications including pharmaceutical research and development, pharmaceutical production, therapeutic monitoring, the generation of plasma and urinary data for pharmacokinetic evaluation, forensic science, toxicology and drug metabolism studies.

In the pharmaceutical field, major applications of TLC include the stability testing of drugs in their various formulations and under extremes of temperature, humidity and light. TLC is also much used in the determination of purity and for quality control.

The development of TLC as a quantitative technique capable of measuring drugs in plasma and urine down to the low ng ml^{-1} level under favourable circumstances has resulted in its application to biomedical analysis for the acquisition of pharmacokinetic data and for therapeutic monitoring. The technique can also be used as a rapid screen to assess patient compliance in clinical trials by providing a simple and inexpensive qualitative assay for the drug in urine. In this laboratory such an approach has been used to determine not only the extent of patient compliance during clinical trials on a novel non-steroidal anti-inflammatory drug, but also the degree to which other anti-inflammatory drugs, such as aspirin, were being taken [4]. Such data is easily obtained and greatly aids the analysis of clinical data. Very often such a compliance assay can be made simple enough to be performed by the clinician in the absence of elaborate facilities. It is difficult to envisage a similar application using HPLC, and indeed to use HPLC for this type of qualitative analysis would be a waste of resources.

TLC is extensively employed in the allied fields of toxicology and forensic science for the rapid identification of drugs and poisons in extremely varied samples ranging from proprietary preparations, illicit materials, body fluids and stomach contents. The extent to which TLC is applied to this area is perhaps best illustrated by a recent article in which chromatographic data was provided for nearly 800 drugs in eight different solvent systems [5].

The simplicity and robustness of TLC has also ensured its continued application to drug metabolism studies. It is particularly useful in the early stages of this type of work where a rapid assessment of the extent of metabolism and an indication of the number and nature of the metabolites is required. Such studies are greatly simplified by the use of radiolabelled test compounds which allow for the specific detection of drug-related compounds in the presence of large amounts of endogenous contaminants. Thus, even though the samples to be analysed include plasma, urine, milk, bile and extracts or homogenates of faeces and tissues, sample preparation can be kept to a minimum. Apart from this, TLC has the advantage that it is possible to locate all the radiolabel which has been applied to the plate, and a complete picture of the distribution of radioactivity in the sample is obtained, regardless of its chromatographic properties. In this way problems of the "loss" of radioactivity due to its non-elution from an HPLC column are avoided.

The introduction of HPLC, especially preparative scale HPLC and gradient elution, into the field of drug metabolism has resulted in a decrease in the use of TLC, especially for metabolite isolation. However, TLC shows no sign of being completely replaced by HPLC, and remains eminently suited to providing a rapid indication of the extent of metabolism which has occurred, and for showing differences due to strain, sex or species.

Recent Developments in Thin-Layer Chromatography

TLC plates

TLC has traditionally been performed on layers formed from silica gel, kieselguhr, alumina, cellulose or polyamide coated onto glass, plastic or aluminium foil. For silica gel, the most popular TLC adsorbent, the thickness of the layers is generally about 0.2 mm for analytical work, and up to 2 mm for preparative plates. The particle size of the silica used for TLC is generally *ca* 20 μm. Two recent developments which have had a significant impact on the practice of TLC have been the introduction of high-performance TLC (HPTLC) and the commercial availability of a range of alkyl-bonded reversed-phase (RP) TLC plates.

HPTLC differs from conventional TLC in a number of respects, the foremost of which is the size of the particles forming the layer. These are in the size range 5–10 μm and are coated in 100–250 μm thick layers. The small size and uniform nature of the particles result in a more rapid and efficient separation than is possible with 20 μm or larger particles.

A number of claims have been made for HPTLC (compared to TLC) including improved resolution, increased number of samples per plate, increased sensitivity (due to smaller spot size), improved reproducibility and performance approaching that of HPLC. However, some of these advantages are more apparent than real. For example, the enhancement in sensitivity claimed for HPTLC is often only 2–3 times that of TLC because of limitations in the amount of sample which can be applied to the HPTLC plate. Good sample application is critical in order to obtain the best performance from HPTLC plates and spots must be kept to 1–2 mm in diameter. Whilst this is still well within the capabilities of most chromatographers it results in sample application being time-consuming and tedious. However, the development of automatic sample spotters (discussed in the next section) and HPTLC plates incorporating a non-adsorbant preconcentration zone has gone some way to minimizing this problem.

Many manufacturers now produce HPTLC plates but these must be selected with care

as the quality of the plates, vital if true HPTLC is to be performed, can leave much to be desired.

There is no doubt that the development of HPTLC has done much to transform TLC from a qualitative into a quantitative technique; however, this has to some extent been at the expense of the simplicity and robustness which are claimed to be features of TLC.

Whilst reversed-phase TLC using stationary phases impregnated with paraffin or octanol has been known for many years, it is only since the introduction of bonded phases that RPTLC has become popular. This new-found interest in RPTLC undoubtedly owes much to the greater understanding of reversed-phase chromatography which has been brought about by the development of RPHPLC. A similar (albeit less extensive) range of stationary phases to that found in HPLC is available for TLC, including C_2, C_8, C_{12}, C_{18} and aminopropyl-bonded silicas. Whilst all of the commercially available bonded RPTLC plates are suitable for RPTLC, it should be noted that nominally similar plates from different manufacturers can have quite different characteristics. In our own studies [4, 6] we have noted marked differences in the chromatographic properties, hydrophobicity and sample capacity of C_{18} bonded RPTLC plates from three different manufacturers. Brinkman and De Vries have performed an extensive evaluation of the different types of RPTLC plate currently available [7].

Experience with bonded RPTLC plates in this laboratory has revealed a number of advantages and disadvantages associated with their use. One of the major problems is the intensely hydrophobic character of many of the plates. This property severely limits the amount of water which may be used in the mobile phase, restricting the range of solvent systems possible, and makes the direct application of aqueous samples (e.g. bile, urine or plasma) impossible. To some extent this has been overcome by the introduction of a number of non-hydrophobic plates (C_8, C_{12} or C_{18} bonded). We have found such non-hydrophobic plates to possess many practical advantages over their hydrophobic counterparts. For example, with the non-hydrophobic type of plate the use of any proportion of water in either solvent or sample is possible [8–10]. Further, the rate of migration of the mobile phase is rapid and independent of the solvent composition. This is contrary to the situation observed with the hydrophobic plates where solvents containing a large proportion of water (eg $\leq$ 40%) give very long development times ($\sim$1–2 h).

Advantages of RPTLC include good chromatography of polar compounds (although ionic compounds such as acids may require the use of ion-pair reagents), improved stability of compounds which decompose on TLC using silica gel [11], high recoveries of material applied to the plate [6, 12] and the ability to develop solvent systems in a logical and predictable way. The latter property results from the linear relationship, generally observed in RPTLC, between R_f and the organic modifier content of the mobile phase. This is illustrated in Fig. 1 for two non-steroidal anti-inflammatory drugs. This predictable relationship between solvent composition and R_f allows the required R_f for a particular compound to be achieved in the minimum number of experiments.

Although there are at first sight many similarities between RPTLC and reversed-phase HPLC it would be wrong to assume that this is always the case. Indeed, there are several examples of factors which are important in HPLC having little or no effect in RPTLC. For example, control of solvent pH is widely used to obtain retention of ionizable compounds in HPLC, but in many cases has no effect in RPTLC [13]. Similarly, pH is of great importance in ion-pair HPLC, but a range of pHs from 2 to 10 had no effect on the R_f values of a range of strongly acidic compounds in ion-pair RPTLC [13].

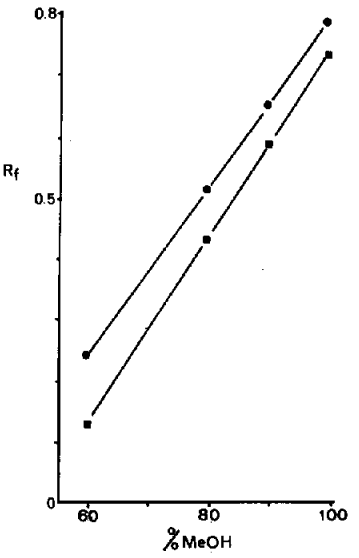

Figure 1
The relationship between R_f and solvent composition for the aryl acetic acids isoxepac (■) and ibufenac (●) on Merck C_{18} bonded, non-hydrophobic, TLC plates.

In ion-pair RPTLC it is important to note that, at least with the longer chain ion-pair reagents (e.g. cetrimide) prior impregnation of the plates with the reagent is essential to obtain efficient ion-pairing [13, 14]. Even with the shorter chain reagents (e.g. tetramethylammonium), which may be used with the reagent present only in the mobile phase, pre-chromatographic impregnation of the plate results in a higher degree of ion-pair formation (lower R_f values) [13].

It was stated earlier that RPTLC was originally performed on silica gel impregnated with paraffin. As a result of the introduction and general availability of alkyl-bonded phases, RPTLC on impregnated plates seems largely to have been ignored. However, the impregnated plates can give just as good chromatographic results as their bonded equivalents, are simple to prepare and are very economical. Further, such plates show many of the advantages of the non-hydrophobic-bonded RPTLC plates in that they may be used for entirely aqueous samples or mobile phases [4, 10].

Development techniques

Conventional TLC involves a single development in a suitable solvent system. This is achieved by immersing the first few millimetres of the adsorbent in the solvent, which is then allowed to migrate far enough up the plate to achieve the desired separation. However, this simple procedure is often insufficient when the separation of complex mixtures is required, especially when the components cover a range of polarities. To overcome this type of problem a number of additional techniques have been devised. These newer methods include continuous development, multiple development, continuous mulitiple development, programmed multiple development (PMD), over-pressurized TLC (OPTLC), circular (or radial) and anticircular development, two-phase–two-dimensional development, and triangular TLC. The essential features of each of these techniques are described below.

Continuous development. For continuous development (CD) the solvent is allowed to advance a fixed distance up the plate at which point it is continuously evaporated [15]. The technique exploits the fact that in TLC the migration velocity of the mobile phase is

inversely proportional to the square of the distance travelled. Consequently the use of short development distances, where the solvent velocity is at its highest, permits separations to be achieved rapidly, especially when combined with HPTLC. The use of solvents of eluotropic strength lower than that required for conventional TLC is also permitted, and may be exploited to increase the selectivity and resolution of the system.

Multiple development. In multiple development (MD) the TLC plate is repeatedly redeveloped, with or without a change in the solvent system. In this way the components may be separated a few at a time, using different solvent systems to accommodate a wide range of polarities. Alternatively a solvent of lower than normal eluotropic strength may be used to increase the separation between spots. A further benefit of the method is that with each development there is a reconcentration effect which maintains a sharp spot profile and thus improves sensitivity.

By combining continuous and multiple development into continuous multiple development (whereby the plate is repeatedly dried during the continuous development) the advantages of both techniques may be combined [15].

Programmed multiple development. As its name suggests, programmed multiple development (PMD) is an instrumentalized form of multiple development [16]. In PMD the plate is automatically cycled though a succession of solvent developments. At the end of each development solvent is removed, using either radiant heat or an inert gas. At each development the solvent front is allowed to migrate slightly further up the plate than the preceding one, until the required separation is achieved. Because of the automatic nature of the system, and the rapid removal of solvent by heating, PMD is less time-consuming and labour-intensive than conventional multiple development, and allows many more developments of the plate. This makes the technique particularly useful for complex mixtures or those requiring the high sensitivity resulting from the spot reconcentration effects observed with each development. The principal limitation of PMD (apart from the cost of the equipment) appears to be the possible thermal breakdown of heat-sensitive compounds during the solvent evaporation steps. Although gas alone may be used to remove solvent, some broadening of the spots is possible [16].

Overpressurized TLC. In most TLC separations the plate is exposed to the vapour of the solvents in which it is developed, with considerable consequences for the chromatography, but this is not the case in overpressurized TLC. Instead the thin layer is completely covered by a flexible membrane, held in place by external pressure [17]. Thus the vapour space above the plate is virtually eliminated. The solvent is introduced to the layer by a pump (to overcome the overpressure on the layer). The balance between the input pressure of the solvent, which increases linearly with increasing solvent migration distance, and the external pressure on the membrane (which must always be higher) has to be carefully maintained. The chromatograms obtained are similar to those in TLC and HPTLC, but with shorter development times due to the constant flow of solvent.

Circular (radial) and anticircular TLC. In circular development the solvent is fed into the centre of the plate and migrates outwards towards its edges. The samples to be analysed are arranged in a circle around the site of solvent entry. Proponents of circular TLC claim that the combination of highly efficient HPTLC plates and circular development results in increased resolution of compounds of low R_f (4–5 times that of

conventional TLC), and faster development times. The latter property results from the optimum separations being achieved in 20–25 mm compared with 40–50 mm for linear development. Much of the recently reported work in circular TLC has been performed using the "U" chamber developed by Kaiser [18]. In this system the developing solvent is fed into the centre of the plate (which is positioned with the adsorbent layer face down) using a motor-driven syringe. The advantage of the "U" chamber over other methods for circular development is the high degree of control which can be exercised over factors such as flow rate, humidity and the composition of the vapour phase close to the adsorbent.

In anticircular development the solvent flows from the edges of the plate inwards to the centre. Samples are therefore applied in a circle of slightly smaller radius than that of the solvent feeding line. Compared with circular development, anticircular TLC offers advantages for the separation of compounds of high R_f.

Kaiser has compared circular, anticircular and linear TLC for a range of chromatographic properties [19]. Anticircular TLC was found to be superior in terms of sensitivity, number of samples per plate, speed of analysis and solvent consumption. Conventional linear TLC was ranked second and circular TLC last. However, the separation number achieved by circular TLC was better than for either of the other two techniques.

Both circular and anticircular development modes require specialized apparatus. Issaq has described a form of TLC which used plates cut into triangles [20]. Samples are spotted along the base of the triangle, and during chromatography (which is accomplished in a standard TLC tank) the shape of the plate results in restricted diffusion of the spots, and hence greater sensitivity. The technique is claimed to combine many of the advantages of anticircular and linear TLC.

Two-dimensional–two-phase TLC. Two-dimensional TLC, reviewed by Azkaria *et al.* [21], is a long established method for the separation of complex mixtures. In the past it has been usual to use the same underlying separation mechanism in each diversion (two consecutive normal phase developments) and change the selectivity of the solvent system to effect resolution. The resurgence of interest in reversed-phase TLC has resulted in the development of a number of two-phase–two-dimensional steps systems employing both reversed- and normal-phase separations. The systems which have been used have been silica gel TLC plates with a strip of silanized (usually C_{18}) material down one edge (of the type manufactured by Whatman or prepared in the laboratory [22]), or the impregnation of the plate with paraffin (or some similar substance) during or after the first normal phase development [23]. Such two-phase systems can be of great utility where both polar and non-polar components must be separated.

Instrumentation

One of the consequences of the introduction and development of HPTLC has been the requirement for careful control at all stages of the TLC procedure (i.e. sample application, development and quantification) in order to obtain the maximum resolution, accuracy and precision of which the new plates are capable. This requirement has led to the instrumentalization of TLC to the extent that virtually the whole TLC procedure can now be automated to a greater or lesser extent. The automated development techniques, such as OPTLC, PMD and the "U" chamber, have been described in preceding sections and will not be discussed further.

In the area of sample application, of critical importance for good HPTLC, devices are now available which can apply volumes of ≥50 nl with a precision of about 1%. Samples are applied either singly or simultaneously (depending on the techniques), from fixed volume capillaries, from a micrometer-controlled syringe, by contact spotting or by spraying. The latter technique, exemplified by the Linomat III (Camag), is especially useful for the application of relatively large volumes (2–99 μl) as bands rather than spots. This type of sample application can give better resolution than spot applications, and can improve quantification by eliminating certain types of systematic error in the measurement of absorbance. This is a result of the more uniform distribution of material within the band, even after chromatographic development, compared with the results observed when the sample is applied as a spot [24].

The TLC UV/visible scanners currently available for the post-chromatographic quantitative evaluation of TLC plates are capable of measuring absorbance, fluorescence, fluorescence quenching (by reflectance), and many are capable of providing the spectra of individual spots *in situ*. The latter capability is especially useful for confirmation of identity, and for determining the optimum wavelength for scanning. For example, the Camag instrument illustrated in Fig. 2 will measure absorbance and fluorescence over the wavelength range 200–800 nm, and linear, circular and anticircular chromatograms may all be evaluated.

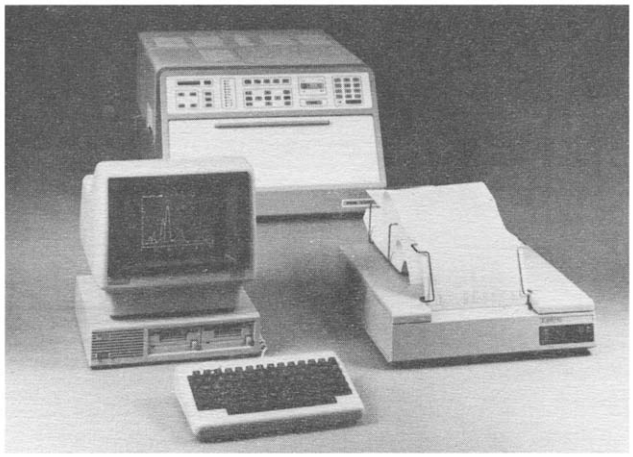

Figure 2
Instrumentation for modern quantitative TLC: the Camag TLC scanner II and data system.

Together with the natural spectroscopic properties of the compounds of interest, there is a vast range of post-chromatographic reagents which may be exploited to provide compound- or class-specific colour reactions in order to enhance specificity and/or sensitivity [25, 26].

Apart from the well established UV/visible absorption and fluorescence detectors a number of other systems exist. For example, the Iatroscan instrument performs chromatography on silica gel coated quartz rods with detection by flame ionization detector (FID). Whilst TLC-FID appears to be a useful innovation as a 'universal detector' for the TLC of carbon compounds, there are reports of problems with reliable quantification [3, 27]. It will be of interest to see if these can be overcome.

Other spectroscopic techniques have also been applied to TLC. For example, mass-spectra have been obtained for a variety of compounds on polyamide-coated plates [28]. The mass spectrum of the non-steroidal anti-inflammatory drug isoxepac from such a plate is shown in Fig. 3. The polyamide was removed from the plate and placed in the tip of a quartz probe. The DI mass spectrum was then obtained in the usual way, and is indistinguishable from a normal DI probe spectrum. Other workers have used the technique of fast atom bombardment (FAB) [29] and recently the use of mass spectrometer as a TLC plate scanner has been described [30]. Whilst perhaps not applicable to every compound, TLC-MS may provide a useful alternative to the time-consuming elution and reconcentration steps otherwise required to obtain mass spectra of compounds on TLC plates.

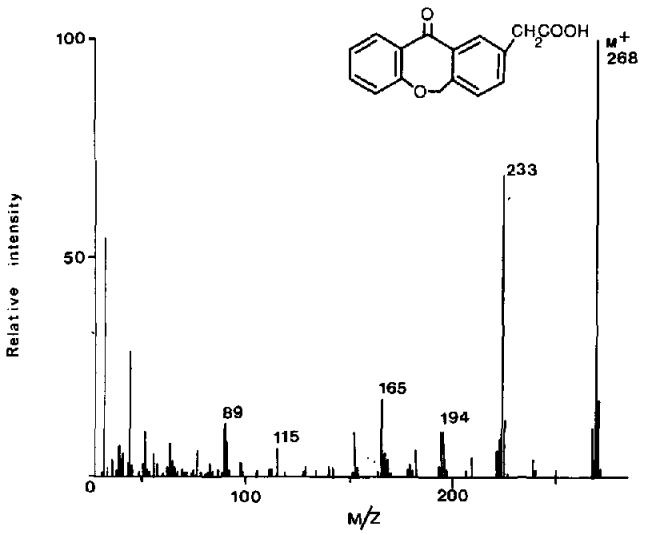

Figure 3
DI probe mass spectrum (70 eV) of isoxepac obtained directly from polyamide TLC plates. The appropriate area of the plate was removed from the TLC plate and placed in the probe tip. A mass spectrum was then obtained in the usual way.

Similarly, the technique of infrared (IR) spectroscopy has been applied to TLC. A recent innovation in this area has been the use of diffuse reflectance Fourier transform infrared spectroscopy enabling good IR spectra to be obtained for microgram amounts of sample on the TLC plate [31].

In the area of drug metabolism one of the most significant developments has been the development of the 'linear analyser' type of radioactivity monitor. This type of instrument differs from conventional radio-TLC detectors of the 'spark chamber' type in that the detector itself does not scan the plate. Instead the ionization chamber is long enough to cover the whole of the area to be scanned (25 × 1.5 cm), and incorporates a position sensitive secondary detector to localize the radioactivity [32]. This type of detector is very sensitive compared with conventional radio-TLC scanners, and can detect as little as 100 dpm of ^{14}C or 1000 dpm of ^{3}H. This greatly increases the speed with which results may be obtained compared with either autoradiography or plate segmentation followed by scintillation counting. A typical instrument, the Berthold LB 284, is shown in Fig. 4.

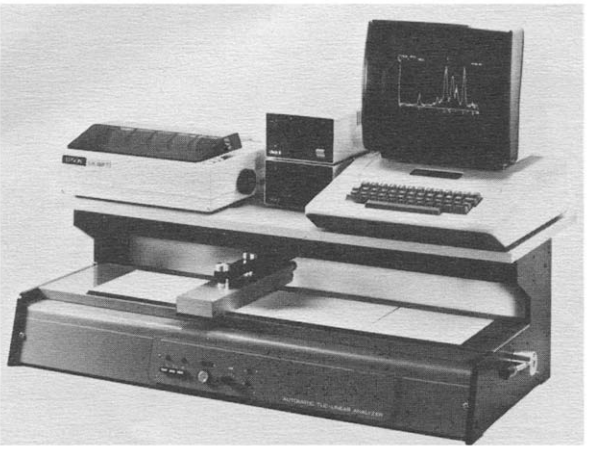

Figure 4
The Berthold LB 284 linear analyser and data system for the detection of radioactivity on TLC plates.

Conclusion

The last decade has seen TLC transformed from a low resolution qualitative technique into a modern high-performance quantitative technique, albeit at the expense of much of its traditional simplicity and cheapness. Given the continuing high level of innovation in both TLC plates and detectors, the future of TLC seems assured.

In its routine role of qualitative analysis it is difficult to envisage a more suitable technique than TLC. However, in the area of quantitative analysis it would seem likely that the dominant techniques will continue to be gas and high-performance liquid chromatography. These techniques are so well established that even the advances which have been made in instrumentalized HPTLC are unlikely to dislodge them. Proponents of quantitative TLC who foresee a revolution in analysis brought about by instrumentalized TLC ignore the large investment which many laboratories have made in time, money and expertise in HPLC and GLC.

Even so, for many quantitative applications instrumentalized HPTLC provides a viable alternative to other systems and the consideration of its adoption as an additional analytical technique has never been more worthwhile.

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